TRANSCRIPTIONAL REGULATION OF SRC BY THE SP FAMILY OF FACTORS AND HISTONE DEACETYLASE INHIBITORS

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ABSTRACT

The SRC gene encodes $pp60^{c\text{-}Src}$, a 60 kDa non-receptor tyrosine kinase that is frequently activated and/or overexpressed in many cancers including colon cancer. In a subset of colon cancer cell lines, it has been shown, that the overexpression of c-Src can be explained, in part, by the transcriptional activation of the SRC gene. As a result, the general goal of this thesis was to further characterize how SRC is transcriptionally regulated in human cancer cell lines. Two highly dissimilar promoters, the housekeeping-like SRC1A promoter, as well as the HIF-1α regulated tissue-specific SRC1α promoter, regulate SRC expression. hnRNP K and the Sp family of factors regulate the SRC1A promoter; however, the true impact of Sp3 on SRC1A activity was not understood. In this thesis, a comprehensive analysis of the effect of Sp3 on SRC1A activity was performed. Physiologically, Sp3 exists as four translational isoforms that, in part, dictate the activation potential of Sp3. In general, the longer forms of Sp3 were modest transcriptional activators of the SRC1A promoter whereas the shorter forms were unable to activate the SRC1A promoter. An analysis of all Sp3 isoforms identified that the shorter Sp3 isoforms could be converted into transcriptional activators of SRC1A if the SUMOylation of a critical lysine residue within the inhibitory domain was prevented. Conversely, SUMOylation of the same isoform had little effect on the activation potential of the longer Sp3 isoforms at the SRC1A promoter. These results suggest that transcriptional activation by Sp3 is promoter context-, isoform- and modification-dependent.

SRC is transcriptionally repressed by histone deacetylase inhibitors (HDIs) and despite unsuccessful studies attempting to identify HDI-responsive elements within the SRC promoter regions none could be identified. This finding also suggests that histone deacetylases (HDACs) may be required for SRC expression. Historically, it was believed that HDIs act at the histone level to alter chromatin dynamics through the inactivation of HDACs to result in histone hyperacetylation and increased transcriptional activation. As such, a systematic investigation of the changes in histone H3 and H4 acetylation status at the transcriptionally repressed SRC promoter regions and the transcriptionally activated $p21^{WAF1}$ promoter region was performed. The $p21^{WAF1}$ promoter was used as control in this study as $p21^{WAF1}$ is a classical example of

a gene transcriptionally activated by HDIs. Interestingly, similar changes in histone acetylation at the $p21^{WAF1}$ promoter and both SRC promoter regions were observed. Upon closer examination of acetylation changes at discreet histone residues, it was observed that in the rare case that a particular residue was differentially acetylated upon treatment at the promoter regions analyzed, the SRC1α and $p21^{WAF1}$ promoter regions demonstrated more similar changes in acetylation as compared to SRC1A. Taken together, these results suggest that histone acetylation status is not an accurate indicator of transcriptional activity following HDI treatment. To further investigate HDI-mediated SRC repression, RNA Pol. II occupancy at the promoter and regions downstream of the promoter were assessed. Despite the continued occupancy of RNA Pol. II at the promoter regions, RNA Pol. II was lost from the 3' UTR upon treatment with HDIs. These findings suggest that RNA Pol. II . may be sequestered at the promoter regions upon treatment with HDIs possibly as a result of impeded transcription initiation and/or elongation. Further analysis of the phosphorylation status of RNA Pol. II identified that transcriptional initiation was indeed occurring despite HDI treatment; however, productive transcriptional elongation could not be confirmed thus suggesting a role for abrogated elongation in HDI mediated SRC repression. Complimentary analysis of the effects of HDACs on SRC expression suggested that while class I HDACs abrogated SRC expression, class II HDACs were required for the maintenance of SRC transcript levels in a promoter-independent fashion. Together, these results provide the basis for a model whereby HDIs repress SRC transcriptional expression through the inhibition of class II HDAC activity to eventually result in curtailed SRC transcriptional elongation.

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alpha-ENaC2	alpha-epithelial Na channel 2
ATF2	activating transcription factor 4
ATP	adenosine triphosphate
AKAp95	A-Kinase-Anchoring Proteins AKAP95 and HA95
AML	acute myeloid leukemia cells
AN-9	butanoic acid prodrug pivaloyl oxymethy butyrate
BA	butanoic acid
BAK	Bcl2-associated K protein
BAX	Bcl2-associated X protein
BCL2	B-cell lymphoma 2
BRE	TFIIB-recognition element
CaMK	calmodulin-dependent protein kinases
CAS	CRK-associated substrate
CAT	Chloramphenicol Acetyltransferase
CBL	Casitas B-lineage Lymphoma
CBP	CREB binding protein
Cdk	cyclin dependent kinase
CE	capping enzyme
C/EBPα	CCAAT/enhancer binding protein-α
ChIP	chromatin immunoprecipitation
CIP	Calf Intestinal Alkaline Phosphatase
CK2	casein kinase 2
CML	chronic myeloid leukemia
Co-REST	co-repressor to RE1 silencing transcription factor
COX	cyclooxygenase
CPSF	cleavage and polyadenylation factor
CRSP	cofactor required for Sp1 activation
c-Src	cellular Src
csk	carboxy-terminal Src kinase
CtBP	carboxy-terminal binding protein

CTD	carboxy-terminal domain
CXCR4	chemokine receptor 4
DMEM	Dulbecco's Modified Eagle's Medium
DPE	downstream promoter element
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DSIF	DRB sensitivity inducing factor
ECM	extracellular matrix
EEC	early elongation complex
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELL	eleven-nineteen lysine-rich in leukemia
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	extracellular signal regulated kinase
ETS	E26 Transformation-specific Sequence
FACT	facilitates chromatin transcription
FAK	focal adhesion kinase
FBS	fetal bovine serum
FDA	Federal Drug Administration
Gcn5	general control nonderepressible-5
GNAT	Gcn5-related N-acetyltransferase
GTF	general transcription factor
HAT	histone acetyltransferase
HBO1	histone acetyltransferase bound to ORC
HDAC	histone deacetylase
Hda1	yeast histone deacetylase 1
HDI	histone deacetylase inhibitor
HIF-1α	hypoxia inducible factor 1α
HNF-1	hepatocyte nuclear factor-1
hnRNP	heterogeneous nuclear ribonuclear protein
HR6A	homologous to RAD6 A
INF-γ	interferon gamma

INO80	inositol biosynthesis 80
Inr	Initiator
ISWI	imitation switch
JMJD2	jumonji C-domain-containing histone demethylase 2
JNK	c-JUN amino terminal kinase
LB	Luria-Bertani
LSD1	lysine specific demethylase 1
MED	mediator subunit
MEF2	myocyte enhancer factor 2
MHC	major histocompatibility complex
Mi-2	Chromodomain 3 for Mi2alpha
MICA	MHC class I protein A
MICB	MHC class I protein A
MMP	matrix metalloproteinases
MORF	MOZ-related factor
MOZ	monocytic leukemia zinc finger protein
MTA-2	metastasis-associated antigen domain
MYST	Moz, YBF2/SAS3, SAS2, Tip60
NAD^+	nicotinamide adenine dinucleotide
NAM	*O*-acetyl-ADP-ribose and nicatinanide
N-CoR	nuclear receptor co-repressor
NELF	negative elongation factor
NES	nuclear export signal
NF-κβ	nuclear factor kappa beta
NKG2D	natural killer cell protein group 2D
NLS	nuclear localization signal
NCP	nuclear core particle
NMT	*N*-myristoyl transferase
NuRD	nucleosome remodeling histone deacetylase
p190 Rho-Gap	p190 Rho GTPase activating proteins
p300	E1A -binding protein of 300 KDa

p21WAF1	21 kDa protein wild-type p53 activated fragment 1
PBS	phosphate-buffered saline
PCAF	p300 /CREB-binding protein-associated factor
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PIC	pre-initiation complex
PP1	protein phosphatase 1
P-TEFb	Positive transcription elongation factor b
PTP	Protein Tyrosine Phosphatase
RECK	reversion-inducing cysteine-rich protein with Kazal motifs
RNA Pol II	RNA Polymerase II
Rpd3	reduced potassium dependency 3
RSV	Rous sarcoma virus
RTK	receptor protein tyrosine kinase
SB	sodium butyrate
SAGA	Spt-Ada-Gcn5-acetylatransferase
SAHA	suberoylanilide hydroxamic acid
SAM	S-adenosylmethionine
SANT	SWI/SNF, ADA, N-CoR and TFIIIB
SCP	small CTD phosphates
SFK	Src family kinases
SH	Src homology domain
SHP	SH2 containing phosphatase
Sir2	silent information regulator 2
siRNA	small interfering RNA
SLIDE	SANT-like ISWI domain
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
Sp1	specificity factor 1
SRC-1	steroid receptor co-activator 1

STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-related modifier
SWI/SNF	switching/sucrose non-fermenting
SWR1	SWI/SNF related
TAF	TBP-associated factor
TBP	TATA binding protein
TFII	transcription factor for RNA polymerase II
Tip60	Tat-interactive protein 60 kDa
TR	thyroid receptor
TRAP	thyroid hormone receptor-associated proteins
TRAIL	TNF-related apoptosis-inducing ligand
TSA	trichostatin A
Ubc-9	SUMO conjugating enzyme
UTR	untranslated region
VPA	valporic acid
VEGF	vascular endothelial growth factor
v-Src	viral-Src

1. REVIEW OF LITERATURE

1.1. Introduction

Eukaryotic transcription is a multifactoral process subject to many levels of regulation. The regulation of chromatin, by a host of pleiotropic enzymes, combined with the tight regulation of transcriptional initiation, elongation and activation leads to the ultimate expression of unique transcripts. The products of the transcripts eventually mediate an array of physiological consequences. The expression of SRC, a proto-oncogene, is subject to regulation by all of these processes. SRC transcriptional regulation is of particular significance as the activation and/or overexpression of $pp60^{c\text{-}Src}$ has been implicated in the development of numerous neoplasms. Furthermore, SRC activity is repressed by a potent class of chemotherapeutic agents, histone deacetylase inhibitors (HDIs), by an unknown mechanism. Therefore, the general goal of this thesis is to investigate the transcriptional regulation of SRC in human cancer cells and to determine the mechanism by which SRC is repressed by this chemotherapeutic class. The following review will summarize the current literature describing transcriptional regulation in a chromatin context, as well as introduce the human SRC gene and the cellular effects -mediated by $pp60^{c\text{-}Src}$.

1.2. Dynamic Chromatin

1.2.1. Chromatin Structure

The organization of the eukaryotic genome has evolved to accommodate the packaging of more than one metre of DNA into the narrow confines of the nucleus. Furthermore, DNA accessibility is maintained such that, replication, transcription and repair occur in a timely fashion (Peterson and Laniel, 2004; Horn and Peterson, 2006). To allow such processes to occur in eukaryotes, a unique packaging system evolved whereby both the structural and functional requirements of DNA packaging are met through a DNA-nucleoprotein complex referred to as chromatin (Horn and Peterson, 2006).

Historically, eukaryotic genomes have been categorized into two distinct cytological classes based on the degree of chromatin compaction exhibited. Nearly 80 years ago, Emil Heitz observed a portion of compact chromatin that persisted throughout the cell cycle in stained moss samples (Zacharias, 1995). Heitz described this condensed chromatin as heterochromatin to contrast with the euchromatin, or "true" chromatin, that underwent repeated phases of decondensation throughout the cell cycle (Dillon, 2004). It is now well established that euchromatin is gene rich, contains the majority of transcribed genes, and replicates early in the cell cycle (Santos-Rosa and Caldas, 2005). In contrast, heterochromatin is considered transcriptionally silent, rich in repetitive sequences, essential for the formation of chromosomal structures, such as centromeres and telomeres, and typically replicates late in the cell cycle (Santos-Rosa and Caldas, 2005; Horn and Peterson, 2006).

1.2.1.1. The Nucleosome

The fundamental unit of chromatin is the nucleosome core particle (NCP). Each NCP subunit is composed of an octamer of highly basic core histone proteins consisting of two of each of the histone proteins: H2A, H2B, H3 and H4. The NCP has a molecular weight of 210 KDa that also includes 147 bp of DNA looped in 1.7 left-handed superhelical turns around the nucleosome core (Arents, *et al*., 1991, Luger, *et al*., 1997). The binding of DNA to the NCP is achieved through the interaction of the histone proteins and DNA at fourteen unique sites (Luger and Richmond, 1998). Histone proteins are highly conserved and contain a globular C-terminal domain that functions

in histone-histone and DNA-histone interactions as well as a critical protruding N-terminal tail (White, *et al*., 2001) (Figure 1.1). The N-terminal tail is prone to a variety of post-translational modifications, including acetylation, methylation, ubiquitynation, SUMOylation and phosphorylation. These modifications are instrumental in multiple key protein-protein interactions. Importantly, the interaction of the NCP and DNA can also be easily altered through the introduction of modified histone proteins into the octamer that differ from the archetypal histone protein described above. These histones are known as histone variants and are suggested to have specific roles in DNA damage response, gene activation and DNA replication (Kober, *et al*., 2004; Kush, *et al*, 2004; Tagami, *et al*., 2004)

Each NCP is separated by 10-60 bp of DNA that interacts with histone H1 (Hansen, *et al*., 1998). Histone H1 is structurally dissimilar to the NCP histone proteins as it is composed of unstructured C- and N-terminal domains linked via a globular helix domain (Wolffe, 1997). Importantly, both histone H1 and the N-terminal tails of the NCP histones are absolutely crucial for the formation of higher order chromatin structures (Woodcock and Dimitrov, 2001).

1.2.1.2. Higher order chromatin structure

The interaction of several NCPs, DNA and histone H1s results in the highly recognizable "beads on a string" 10 nm fibre. However, this secondary structure is often associated and in dynamic equilibrium with the further condensed 30 nm solenoid fibre *in vitro* (McBryant, *et al*., 2006). Studies using model systems with histones lacking the N-terminal domains have highlighted the critical nature of core histones in 30 nm fibre formation (Carruthers and Hansen, 2000). In particular, the histone H4 N-terminal domain is key in this process due to interactions between this domain and histones H2A and H2B from neighbouring nucleosomes, which aid in chromatin condensation (Dorigo, *et al.*, 2004). The 30 nm solenoid fibre is structurally characterized as containing 6 nucleosomes per 11nm, which is approximately 6 nucleosomes per turn, and has been deemed the primary helix of chromatin (Woodcock and Dimitrov, 2001).

There are many models of how chromatin compaction continues after the formation of the solenoid and an exhaustive review of all models is beyond the scope of this thesis. However, it is worth noting that in all of the models, histone H1 is required

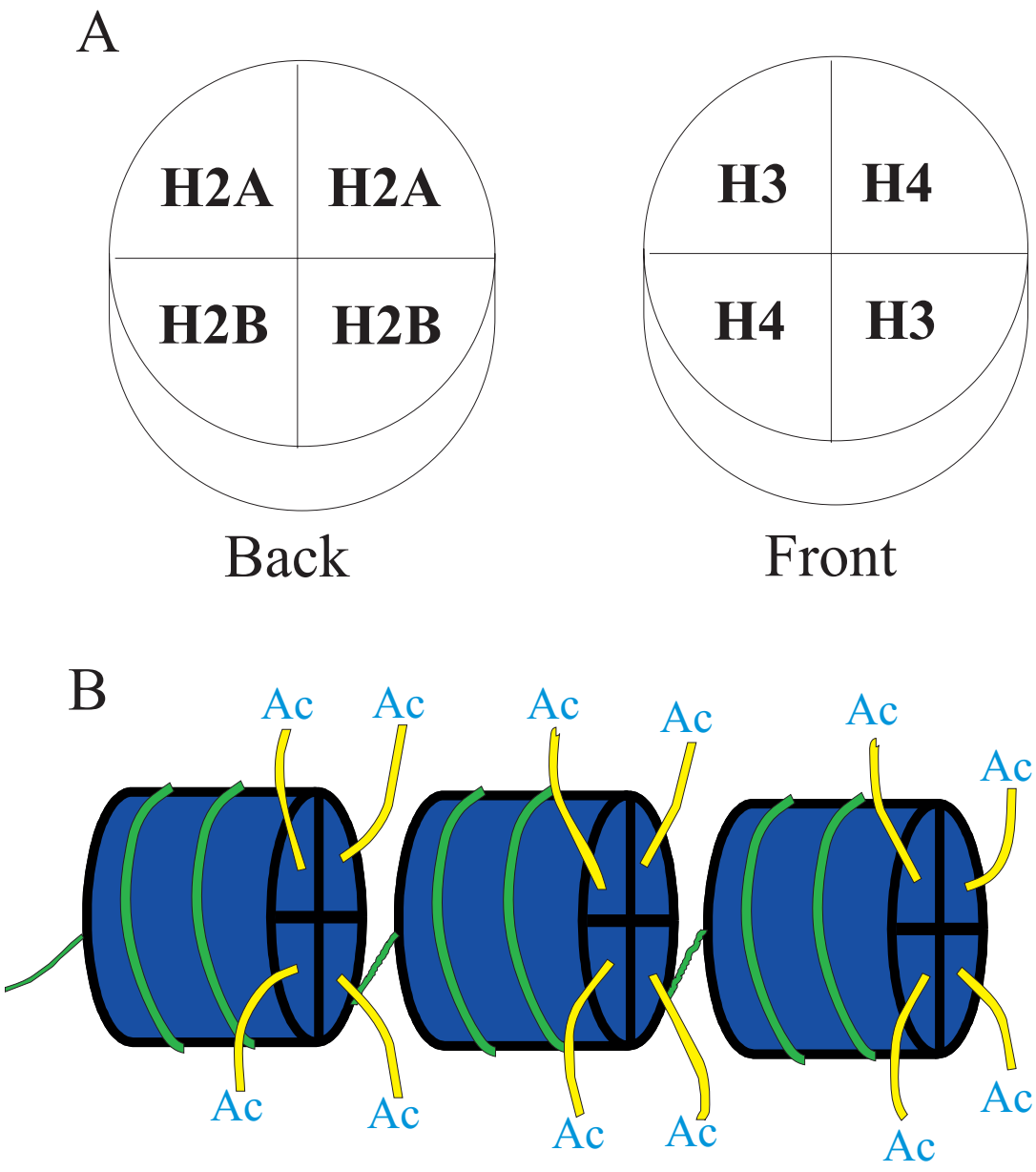


Figure 1.1. The nucleosome core particle (NCP). (A) Schematic representation of a histone octamer. H2A and H2B form dimers that interacts with a H3 and H4 tetramer to form the histone octamer. (B) Several histone octamers connected by DNA (green). Yellow lines represent protruding amino-terminal tails of histones which are host to a variety of post-translational modifications (adapted from Hess-Strump, 2005; **Workman, 2006**).

(Daban, 2003; McBryant, *et al*., 2006). Histone H1 stabilizes oligomeric tertiary chromatin complexes, through electrostatic neutralization of linker DNA segments, including the formation of the intermediate fibres formed from the looped solenoid 30 nm fibres (200-300) (Carruthers and Hansen, 2000; Daban, 2003). The coiled chromatid is subsequently formed from the intermediate fibre (Daban, 2003). Despite years of research, the final steps in the formation of the chromosome are still not well understood. However, recent advances utilizing *Xenopus* eggs and fission yeast have identified that chromatin condensation requires the co-operation of many multi-protein complexes. One such example is the condensin complex, which implicates ATP-dependent enzymes in both chromosome condensation and segregation (Hirano, 2000).

1.2.2. ATP--dependent Chromatin Remodeling Enzymes

The basic requirements for chromatin structure are outlined above. However, for chromatin to be both dynamic and functional a great deal of DNA must be compartmentalized and yet necessary genetic information must remain available for replication, repair and transcription (Horn and Peterson, 2006). For this to be achieved, euchromatin must be able to decondense to allow repair enzymes, transcription and replication factors access to DNA target sequences. ATP--dependent chromatin remodelling enzymes are essential in altering and maintaining the properties of functional chromatin as they are able to physically eject and re-locate nucleosomes from their cognate DNA locations.

Eukaryotes have five classes of nucleosomal remodelling enzymes. The classes include: the switching/sucrose non-fermenting (SWI/SNF) family, the imitation switch (ISWI) family, the inositol biosynthesis 80 containing (INO80) family, the SWI/SNF related factor (SWR1) family and the nucleosome remodelling histone deacetylase/ chromodomain3 for Mi2alpha/ chromodomain (NURD/Mi-2/CHD) family. These enzymes are grouped together by the presence of an ATPase domain and due to their role in mobilizing nucleosomes to different locations along the DNA. The remodelling enzymes are responsible for the spacing of nucleosomes throughout chromatin assembly, as well as allowing transcription factor access to specific sites during transcriptional regulation and the regulated access of DNA-repair factors to damaged

DNA within the chromatin (Becker and Horz, 2002; Saha, *et al*., 2006). The three classes most pertinent to this thesis will be briefly reviewed.

The human SWI/SNF class of chromatin remodellers is involved in a variety of essential cellular processes including transcriptional elongation, cellular signalling and alternative splicing (Zhao, *et al*., 1998; Corey, *et al*., 2003; Batsche, *et al*., 2006). Aside from the ubiquitous ATP-ase domain, the SWI/SNF family contains a unique C-terminal domain and at least one bromodomain that is (are) capable of recognizing acetylated histone residues (Winston and Allis, 1999; Hassan, *et al.*, 2001). The presence of the bromodomain, which recognizes acetylated residues, suggests that remodellers within the SWI/SNF family function primarily to re-organize nucleosomal position to promote transcriptional activation (Martens and Winston, 2003). Interestingly, both the SWI/SNF and ISWI family of remodellers re-organize nucleosomes in a 3'-5' direction despite inherent differences in both the mechanism of nucleosomal translocation along the DNA strand and the effect of the translocation (Saha, *et al.*, 2006). However, the precise mechanisms of the nucleosomal remodelling are beyond the scope of this thesis.

The ISWI family of nucleosome remodellers contain an ATPase domain and C-terminal SANT and SLIDE domains. The SANT domain is important for both DNA and histone-tail binding, whereas the SLIDE domain exclusively binds DNA. The ISWI remodellers are typically associated with transcriptional repression (Goldmark, *et al*., 2000), X-chromosome regulation (Deuring, *et al*., 2000) and most significantly, chromatin assembly following replication (Bozhenok, *et al.*, 2002).

The NURD family of ATP--dependent chromatin remodellers is also frequently associated with transcriptional repression and gene silencing (Wade, *et al*., 1999). The human NURD complex was originally isolated from HeLa nuclear extracts and was subsequently characterized as having multiple methylation recognizing chromodomains, histone deacetylase (HDAC) domains and a metastasis-associated antigen domain (MTA-2), as well as being ATP -dependent (Becker and Horz, 2002). ATP-dependent chromatin remodellers within the NURD family are excellent examples of nucleosome remodelling proteins capable of not only mobilizing nucleosomes, but also recognizing methylated histone residues and deacetylating histone targets. The functional

consequences of these pertinent domains are essential for chromatin function and will be discussed further in the next section.

1.2.3. Histone Modifying Enzymes

In the previous section, a critical component of the chromatin machinery was reviewed. Other major components of chromatin metabolism, which function in concert with the ATP-dependent chromatin remodellers, are histone acetyltransferases (HATs), and histone deacetylases (HDACs). These complimentary classes of enzymes covalently modify lysine residues within histones and other proteins in a manner that directs the action of the ATP-dependent chromatin remodellers.

1.2.3.1. Histone Acetyltransferases (HATs)

The acetylation of core histones by HATs is frequently associated with transcriptional activation. In fact, the first HAT isolated, from *Tetrahymena*, was homologous to the previously characterized yeast transcriptional co-activator Gcn5 (general control nonderepressible-5) (Brownell, *et al*., 1996). Subsequently, many transcriptional co-activators have been discovered to contain HAT activity (Peterson and Laniel, 2004). HATs frequently exist in large multi-protein complexes critical for locus targeting. These multi-protein complexes expand HAT specificity such that particular residues are acetylated at particular genomic loci. Historically, models have suggested that HAT activity mediates transcriptional activation by modifying the innate lysine charge on histone nucleosome tails through acetylation. It was hypothesized that the acetylation of these residues disrupted histone tail-DNA interactions, resulting in an open or "active" chromatin conformation that was permissive to the binding of transcriptional activators (Santos-Rosa and Caldas, 2005). A lack of evidence has since discredited this idea; even the lysine residue rich histone H3 tail would only have a 10-30 % decrease in positive charge if all 13 available lysine residues were acetylated (Peterson and Laniel, 2004). Current dogma now suggests that HATs are frequently associated with transcriptional activation due to their ability to both acetylate lysine residues and recognize acetylated lysine residues via the presence of one or more bromodomains (Mellor, 2006).

Human HATs have been organised into several superfamilies based on sequence similarity and mechanisms of histone substrate binding and catalysis. Human HAT characterization has identified that these HAT complexes share similar composition to known yeast complexes (Sterner and Berger, 2000). The GNAT (Gcn5-related N-acetyltransferase) superfamily includes the potent co-activators GCN5L and PCAF (p300/CBP associated factor). These co-activators share similar carboxyl terminal ends, with each containing a 160 residue HAT domain and conserved bromodomains (Kuo and Allis, 1998). Studies from yeast suggest the GNAT superfamily uses acetyl CoA to specifically acetylate lys13 of histone H3 (H3K9 and 14 in humans) and lys8 and lys16 of histone H4 (H4K8 and 16 in humans) *in vitro* (Kuo, *et al*., 1996) However, *in vivo*, the overexpression of yeast Gcn5p results in the hyperacetylation of all four core histones (Kuo, *et al.,* 1998). This disparity can be explained by the observation that HAT involvement in transcriptional activation is context-dependent and other complex components influence how a HAT will affect a particular substrate (Sterner and Berger, 2000). Therefore, it should be noted that the majority of studies have examined the activity of HATs as complex components rather than the activity of the HAT enzyme itself. In the case of the GNAT superfamily, GCN5 is frequently associated with the potent co-activator SAGA (Spt-Ada-Gcn5-acetylatransferase) complex in yeast (STAGA in humans) and has been implicated in the transcriptional activation of numerous genes (Brownell, *et al*., 1996). In a further example, PCAF interacts with the p300/CBP HAT superfamily and is required for the transcriptional activation of a variety of genes including p73, p53, and ETS responsive genes (Goel and Janknecht, 2003; Zhao, *et al*., 2003).

HATs within the nuclear p300/CBP (E1A binding protein p300/CREB-binding protein) family contain a large 500 amino acid residue HAT domain, a bromodomain and three key cysteine-histidine rich domains (TAZ, PHD, ZZ) that are required for protein-protein interactions (Santos-Rosa and Caldas, 2005). p300/CBP HATs are global modulators of transcription that use acetyl CoA to specifically acetylate histones H3/H4 and histones H2A/H2B (Kuo and Allis, 1998). More specifically, in mammals, histone residues H2AK5, H2BK12, H2BK15, H3K14, H3K18, H3K23, H4K5 and H4K8 are acetylated by one or both of these powerful HATs (Peterson and Laniel,

2004). HATs in this versatile superfamily have also been reported as acetylating non-histone targets such as: p53, viral oncoprotein E1A, androgen receptors and other transcription factors. The effect of this acetylation frequently results in the stabilization of the protein target (Zhang, *et al.*, 2000; An, *et al.*, 2004; Santos-Rosa and Caldas, 2005). Interestingly, PCAF is often in complex with p300 and/or CBP and is also associated with the acetylation of the above non-histone targets.

Finally, the MYST superfamily is unique from the other HAT superfamilies in that it contains a conserved 370 residue MYST domain that acetylates target lysine residues through an acetyl-cysteine intermediate. This unique catalytic mechanism is common to all MYST members, including Tip60 (Tat-interactive protein 60 kDa), MOZ (monocytic leukemia zinc finger protein), MORF (MOZ-related factor) and HBO1 (histone acetyltransferase bound to ORC). Members of this family also contain a cysteine-rich zinc binding domain within the MYST HAT domain and a chromodomain that recognizes methylated residues (Yang, 2004; Santos-Rosa and Caldas, 2005). MYST HATs specifically acetylate histone H2AK5, H3K14, H4K5, H4K8, H4K12 and H4K16 (Peterson and Laniel, 2004). Furthermore, MYST HATs have been implicated in both transcriptional activation and silencing (Yang, 2004). In particular, Tip60 has a role in the transcriptional repression of signal transducer and activator of transcription-3 (STAT3) but also functions as a co-activator for nuclear factor kappa beta (NF-κβ) and c-myc (Baek, *et al*., 2002; Frank, *et al.*, 2003; Xiao, *et al*., 2003).

Notably, aside from the specific HATs mentioned above, many proteins contain HAT domains but are not categorized within the HAT superfamilies. Such examples include the transcription factors TAF1 (acetylates H3K14), ATF2 (acetylates H4K5, K8 and K16), and Elp3 (acetylates H3K14 and H4K8) and the nuclear hormone related SRC-1 (acetylates H3K9, K14) (Peterson and Laniel, 2004; Yang, 2004). It is also important that many HATs or HAT containing proteins have the intrinsic ability to auto-acetylate (Yang, 2004). Specifically, PCAF autoacetylation is required for nuclear localization and TFIIB autoacetylation is required for the formation of the pre-initiation complex responsible for transcriptional activation (Choi, *et al.*, 2003; Santos-Rosa, *et al*. 2003).

1.2.3.2. Histone Deacetylases (HDACs)

The reversible deacetylation of core histone residues is frequently associated with transcriptional repression. This is not surprising considering that HATs are often associated with transcriptional activation and HATs and HDACs have contrary functions. In fact, these enzymes exist in dynamic equilibrium within the cell and the acetylation status of histones and non-histone proteins is governed by the opposing actions of these enzymes. Similar to HATs, historically, it was hypothesized that HDACs functioned by relieving the positively charged lysine residues of the negatively charged acetyl moiety. The restoration of positively charged lysine residues within the histone tails was thought to result in increased nucleosome-nucleosome and nucleosome-DNA interactions thus "closing" the chromatin and preventing access to co-activators (Santos-Rosa and Caldas, 2005). This theory has since been replaced by the idea that a reduction in acetylation reduces the binding of bromodomain containing co-activators, thereby discouraging transcriptional activation. Indeed, it has been demonstrated that non-acetylated histone residues promote the association of repressors (Yang and Seto, 2003).

The four classes of mammalian HDACs are characterized based on homology to yeast HDACs. Class I HDACs possess homology to yeast Rpd3 (reduced potassium dependency 3) and include HDACs 1, 2, 3 and 8. These HDACs are ubiquitously expressed, primarily nuclear and all contain a zinc-dependent HDAC domain. HDAC enzymatic activity is mediated via a charge-relay system whereby nucleophilic attack of the acetyl moiety is mediated by a critical Zn^{2+} ion found in the tubular active site of the enzyme (DeRuijter, *et al.*, 2003; Hildmann, *et al*., 2006). HDAC1 and 2 are highly similar enzymes that only display activity within protein complexes *in vivo*. These complexes modulate the intrinsic HDAC activity and are required for binding to DNA and histone targets. HDAC1 and 2 are found in complex with Sin3, the ATP-dependent remodeller NuRD and co-repressor to RE1 silencing transcription factor (CoREST). Conversely, HDAC3 requires the co-repressors silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (N-CoR) for repressive activity, as both of these co-repressors contain conserved deacetylase-activating domains. HDAC3 also contains a nuclear localization signal (NLS) and

nuclear export signal (NES); thus HDAC3 is the only class I HDAC capable of shuttling in and out of the nucleus (Khochbin, *et al*., 2001; DeRuijter, *et al.*, 2003). In fact, recent reports have suggested that HDAC3 localizes to the plasma membrane where it is a substrate for c-Src (Longworth and Laimins, 2006). HDAC8 is most similar to HDAC3 but is not as well characterized and is generally present in low abundance within the cell (Khochbin, *et al*., 2001; DeRuijter, *et al.*, 2003). As hinted at above, class I HDACs can be regulated by post-translational modifications. HDAC1, 2 and 3 are all phosphorylated by casein kinase 2 (CK2). However, only HDAC3 is phosphorylated by c-Src. HDAC1 phosphorylation results in the disruption of complex formation and loss of enzymatic activity. Conversely, HDAC2 and HDAC3 activity is upregulated by phosphorylation (Pflum, *et al*., 2001; Galanski, *et al*., 2002; Tsai and Seto, 2002; Zhang, *et al*., 2005) Similar to HDAC1, HDAC8 is negatively regulated by cAMP-dependent protein kinase A (Lee, *et al*., 2004).

Class II HDACs include HDAC4, 5, 6, 7, 9 and 10. These HDACs are characterized as being larger in size, more tissue specific than class I HDACs, and can be either nuclear or cytoplasmic. This class is homologous to yeast histone deacetylase 1 (HDA1). Like class I HDACs, the deacetylation activity of class II HDACs is dependent on a Zn^{2+} ion present in the tubular-shaped active site of the enzyme (DeRuijter, *et al.*, 2003). Unlike class I HDACs, however, the nature and activity of a particular class II HDAC is -dependent on it's intracellular localization (Khochbin, *et al*., 2001). As such, the catalytic activity of this class of HDAC is often characterized by the presence of a NLS and/or NES domain. HDAC4, 5 and 7, which contain a C-terminal catalytic domain and N-terminal NLS, are the most similar class II HDACs. Notably, HDAC5 also contains an NES within the catalytic domain and therefore can shuttle in and out of the nucleus. Furthermore, all three of these HDACs contain N-terminal binding domains for the co-repressor C-terminal binding protein (CtBP), transcription factor myocyte enhancer factor 2 (MEF2) as well as 14-3-3 proteins and it is through interactions with these factors that cellular localization is determined (Bertos, *et al.*, 2001; DeRuijter, *et al.*, 2003). For example, it is through the repressive interaction with MEF2 that HDAC4, 5 and 7 are localized to the nucleus where they appear to have a role in muscle differentiation (Lu, *et al*., 2000). Similarly, HDAC4, 5

and 7 are further implicated in transcriptional repression due to complex formation with the co-repressor SMRT/N-CoR. It is through this interaction that these class II HDACs are found in complex with HDAC3 (Bertos, *et al.*, 2001).

Evolutionary analysis suggests that HDAC6 and HDAC10 are the next most similar class II HDACs. However, HDAC6 is unique in that it contains two catalytic domains, a zinc finger motif and a unique SE14-repeat domain that is exclusive to human HDAC6 and essential for cytoplasmic retention (Yang and Gregoire, 2005). HDAC 6 functions primarily as a tubulin deacetylase, involved in microtubule-dependent cell motility. Despite this primary function, HDAC6 is also found within the nucleus in complex with other HDACs (DeRuijter, *et al.*, 2003). HDAC10 contains a N-terminal catalytic domain similar to that of HDAC6. The C-terminal half of HDAC10 contains an NES and putative lysine-rich second catalytic domain. HDAC10 is the least characterized of the HDAC enzymes so very little is known concerning the function of this HDAC. However, HDAC10 has been shown to exist in complex with HDAC1, 2, 3, 4, 5, and 7 thereby suggesting a role for HDAC10 in transcriptional repression (DeRuijter, *et al.*, 2003; Yang and Gregoire, 2005). Finally, HDAC9 exists as at least three splice variants and contains a N-terminal catalytic domain. HDAC9 appears to be expressed primarily in muscle tissue where it also interacts with MEF2 in a repressive fashion similar to that observed with HDAC5 (Chang, *et al.*, 2004; Yang and Gregoire, 2005).

Similar to class I HDACs, class II HDACs are also susceptible to post-translational modifications such as phosphorylation. Calmodulin-dependent protein kinases (CaMKs) phosphorylate HDAC4, 5 and 7 to promote nuclear export (McKinsey, *et al.*, 2000; Yang and Gregoire, 2005). Moreover, HDAC4 can be phosphorylated by extracellular signal regulated kinase (ERK1) and ERK2 *in vitro* suggesting a role for HDAC4 in signalling pathways (Gray and Teh, 2001). HDAC4, 5 and 9 are subject to ubiquitination and SUMOylation, which do not affect nuclear localization (Yang and Gregoire, 2005).

Most significantly, class I and class II HDACs are not always associated with transcriptional repression. HDAC1 is required for the transcriptional activation of STAT5 target genes (Rascle, *et al*., 2003; Xu, *et al*., 2003). HDAC1, 2 and 3 enhance

expression of STAT1-dependent genes (Klampfer, *et al*., 2004). HDAC7 has been implicated in transcriptional activation through its interaction with HIF-1α (hypoxia inducing factor 1α) (Kato, *et al.*, 2004). Moreover, select interferon response genes require HDAC activity to activate transcription (Chang, *et al*., 2004; Sakamoto, *et al.*, 2004). Furthermore, yeast HDACs have also been frequently associated with transcriptional activation. For example, Rpd1 has been reported as being required for maximal transcriptional activation, as well as repression, of a subset of genes in *Saccharomyces cerevisiae* (Vidal, *et al*., 1991). In addition, yeast HDACs Rpd3 and Hos2 are required for the activation of DNA-damage inducible genes RNR3 and HUG1 (De Nadal, *et al.,* 2004; Sharma, *et al*., 2007). Rpd3 has also been implicated in the transcriptional activation of osmoresponsive genes (De Nadal, *et al*., 2004).

The third class of HDACs, the Sir2-like (silent information regulator 2-like) class, is structurally and catalytically different from the first two classes of HDACs. Unlike the Zn^{2+} ion dependency exhibited by the first two HDAC classes, class II HDACs require nicotinamide adenine dinucleotide (NAD^{+}) as a co-factor for deacetylase enzymatic activity. During the deacetylation reaction, NAD^{+} breaks down to produce in *O*-acetyl-ADP-ribose and nicatinanide (NAM) (Santos-Rosa and Caldas, 2005). There are seven human class III HDACs (SIRT1-7) and despite the relatively recent discovery of this class of HDACs it appears that they are functionally distinct from the first two HDAC classes. For example, the NAD^{+} requirement suggests a susceptibility to cellular energy requirements/redox states (Khochbin, *et al*., 2001). Furthermore, in *S. cerevisiae* and *C. elegans*, this class of HDAC has been implicated in the ageing process whereby overexpression of the sirtuens leads to an increase in lifespan (Trapp and Jung, 2005). A comprehensive survey of all class III HDAC family members is beyond the scope of this thesis.

The fourth class of HDACs contains only one member. The function of HDAC11 is relatively unknown. However, this HDAC is nuclear, like class I HDACs, tissue specific, like class II HDACs, and contains an enzymatic domain similar to that seen in both class I and II HDACs. Due to the similarities and differences between HDAC11 and both class I and II HDACs, HDAC11 is considered in a class by itself (Minucci and Pelicci, 2006).

1.2.3.3. Other Covalent Histone Modifying Enzymes

Histone tail residues may also be post-translationally modified by methylation, phosphorylation, SUMOylation and/or ubiquitination. Similar to acetylation, these modifications are frequently associated with a particular chromatin state. These modifications, the enzymes that elicit them and associated functions will be briefly reviewed.

Histone methylation/demethylation occurs at the side chain nitrogen atoms of lysine or arginine residues through the donation of a methyl group from S-adenosyl-L-methionine (SAM) (Gary and Clark, 1998). Histone arginine residues are mono- or di-methylated; the latter in a symmetrical or asymmetrical manner. The degree and manner of histone methylation is -dependent on the methyltransferase involved in the reaction. Generally, histone arginine methylation correlates with transcriptionally active genes. Methylated arginines are susceptible to a deimination reaction thus resulting in the modified residue being converted into a citrulline (Santos-Rosa and Caldas, 2005). Histone lysine residues may be mono, di- or tri-methylated and, like arginine methylation, the lysine residue and degree of methylation is -dependent on the methyltransferase employed. Lysine methylation is associated with a variety of functions including transcriptional activation, chromatin condensation, constitutive heterochromatin formation and transcriptional silencing/repression. Recently, the lysine specific demethylase 1 (LSD1) and jumonji C-domain-containing histone demethylase 2 (JMJD2) families of lysine demethylases, were identified as being capable of reversing lysine methylation (Santos-Rosa and Caldas, 2005; Cloos, *et al*. 2006).

Specific serine and threonine residues on the histone tails are subject to phosphorylation. This type of post-translational modification is reversible and mediated by the interplay of kinases, such as the Aurora-B kinase, and phosphatases, such as protein phosphatase 1 (PP1). Phosphorylation of histone residue targets is frequently associated with the condensation of chromatin to form heterochromatin and yet, conversely, also with the transcriptional activation of select eukaryotic genes (Santos-Rosa and Caldas, 2005; Johansen and Johansen, 2006).

Both ubiquitin and small ubiquitin-related modifier, SUMO, can also modify histone lysine residues. Histone lysine mono-ubiquitination is -mediated primarily through the ubiquitin conjugating enzyme homologous to RAD6 A (HR6A) and, depending on the residue affected, has been associated with euchromatic regions, transcriptional activation, spermatogenesis and meiosis (Osley, 2004). The SUMOylation of histone lysine residues is, however, not as well characterized, although this modification is usually associated with transcriptional repression. The SUMO conjugating enzyme Ubc-9 is required for the SUMOylation of histone lysine residues (Shiio and Eisenman, 2004).

1.2.4. Histone Code

Chromatin is a dynamic environment. The last few sections have outlined several key processes involved in chromatin dynamics. The following section serves to bring these critical components together under the guise of the histone code. The histone code is the cooperation of distinct histone modifications to result in transcriptionally active or inactive chromatin states (Mellor, 2006).

Covalent histone residue modifications have been suggested to be required for the recruitment of essential ATP-dependent chromatin remodelling enzymes and transcriptional regulators to DNA promoter regions (Winston and Allis, 1999; Hassan, *et al.*, 2001). Therefore, these modifications are suggested to act as a type of scaffold for the assembly of large multi-protein complexes that direct or mediate the remodelling of chromatin. Significantly, many of these modifying enzymes, such as HDACs, are actually in complex with the ATP-dependent remodelling enzymes and in some cases require complex formation to be functional (Khochbin, *et al*., 2001; DeRuijter, *et al*., 2003). If this is indeed the case, then covalent histone modifications themselves do not occur prior to remodelling events but may actually occur during or after chromatin reshuffling. Interestingly, many co-activators and co-repressors contain bromo- and chromodomains required to recognise methylated and acetylated residues, suggesting that some degree of modification is likely to occur prior to complex binding. Regardless of the order that these events occur, there appears to be a synergy amongst histone

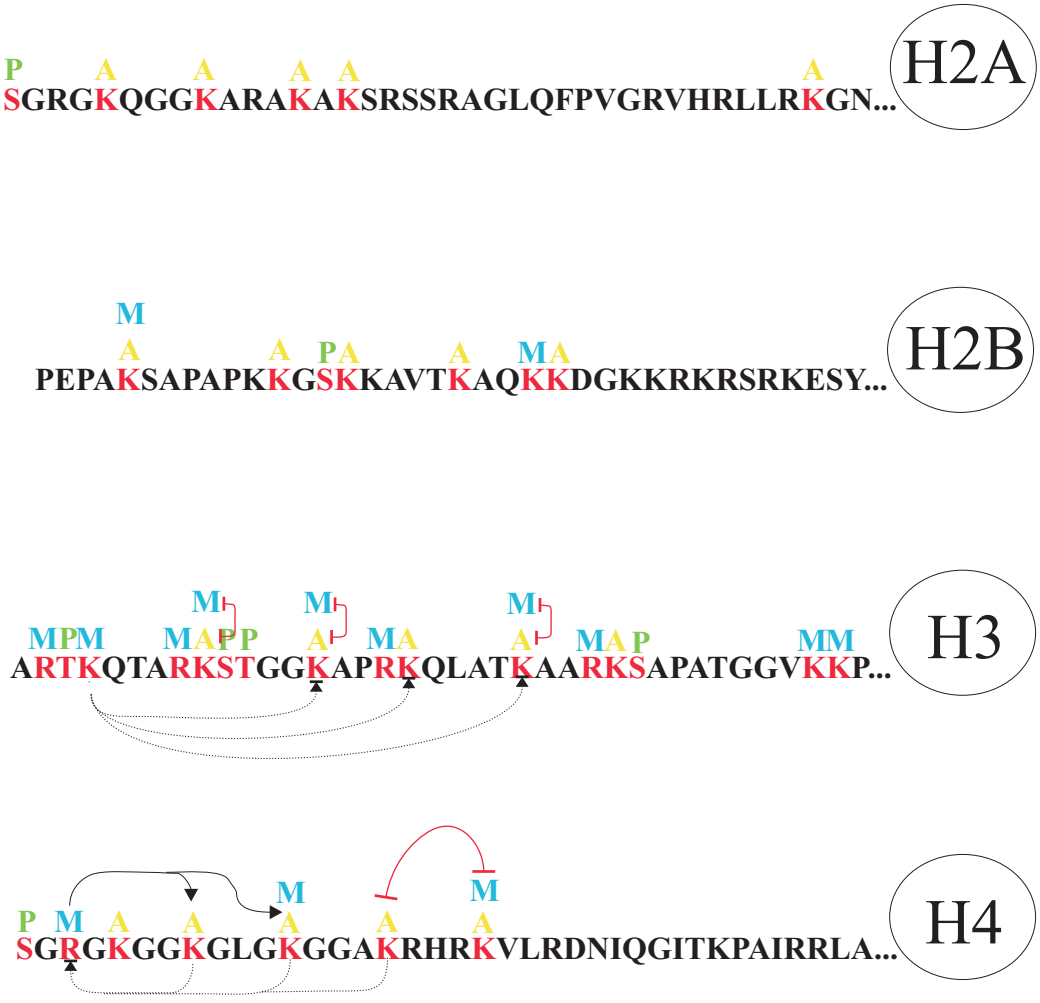


Figure 1.2. The histone code. The residues of amino-terminal tails of histones H2A,H2B, H3 and H4 are shown. All residues in red are subject to post-translational modification. P represents phosphorylation, M represents methylation and A represents acetylation. The ⇢ indicates the ability of one modification to result in the removal of the other modification. ⊣ indicates that a modification negatively impacts the other modification. → indicates the addition of one modification is a result of another modification.
(adapted from abcam.com/chromatin/posters)

modifications that can be suggestive of a transcriptionally active or repressive state (Jenuwein and Allis, 2001). A particular modification may indicate a transcriptionally active genomic region or may serve to influence other modifications to exact alternate effects (Figure 1.2.).

For example, the combination of H4K8, H3K14 acetylation and H3S10 phosphorylation is frequently associated with a transcriptionally active chromatin region. Conversely, H3K9 trimethylation prevents H3K9 acetylation and is associated with a repressive chromatin state (Peterson and Laniel, 2004). Methylation on H4R3 is suggested to facilitate acetylation of H4K8 and H4K12, modifications often associated with active chromatin (Zhang and Reinberg, 2001). Significantly, H4K16 acetylation is credited with transforming a repressive genomic region into an active region because this modification precludes the binding and remodelling activities of ISWF, thus providing an example that the effect -mediated by one modification is not always reliant on other histone modifications (Saha, *et al*., 2006; Shia, *et al*., 2006). Finally and most importantly, it should be noted that a particular modification or combination of modifications is(are) not always predictive of a particular gene-specific outcome.

1.2.5. Histone Deacetylase Inhibitors

Many of the studies involving chromatin remodelling and the histone code have been aided by the use of a particular new class of promising anti-neoplastic agent, known as histone deacetylase inhibitors (HDIs). As the name suggests, these drugs specifically inhibit class I, II and IV HDACs. The treatment of cells with these drugs has far-reaching cellular consequences.

Of primary importance to this thesis, inhibition of HDACs upsets the dynamic equilibrium of acetylation and deacetylation within the cell. Because HDACs, and deacetylation, are frequently associated with transcriptional repression, it was expected that there would be an overall increase in transcriptional activation post treatment. This was not the case. Data from microarray studies suggest that anywhere between 2% and 22% of all genes are affected by HDIs (Van Lindt, *et al*., 1996; Glaser, *et al*., 2003; Mitsiades, *et al*., 2003; Gray, *et al*., 2004; Peart, *et al*., 2005). While discrepancies obviously exist in the reported numbers of affected genes, most significantly, equal

numbers of affected genes appear to be up or down -regulated in these studies. Such findings support the now pervading sentiment, that genes are both activated and repressed by HDIs.

The differential transcriptional regulation caused by HDIs has a significant impact on the cell. The HDI -mediated, p53 -independent, transcriptional upregulation of $p21^{WAF1}$, and/or the simultaneous downregulation of cyclins A and D, can induce cell cycle arrest at the G1/S boundary (Richon, *et al*., 2000; Sandor, *et al*., 2000). HDIs also transcriptionally activate TNF-related apoptosis-inducing ligand (TRAIL) and FAS resulting in apoptosis through the death-receptor pathway. Similarly, HDIs may also induce apoptosis via the upregulation of pro-apoptotic BCL2 proteins (BAK/BAX) and the downregulation of anti-apoptotic BCL2 family members (BCL2/BCL-X_L), thereby facilitating the activation of the caspase -dependent mitochondrial apoptotic pathway (Green, 2000; Johnstone, 2002).

More recent findings indicate that HDIs are capable of interfering with existing phosphatase complexes. Specifically, it has been demonstrated that HDIs are capable of blocking PP1/HDAC1/6/10 complex formation (Brush, *et al*., 2004). The abrogated interaction of PP1 and HDAC1/6 results in increased interaction between Akt and PP1, thus decreasing Akt phosphorylation and lowering the apoptosis threshold (Chen, *et al*., 2005). Subsequent studies have identified that in breast cancer cells the decreased Akt activity leads to increased susceptibility to apoptosis due to heightened glycogen synthase kinase 3 beta (GSK3beta) activity (Alao, *et al*., 2006). The interaction of HDAC3 and A-Kinase-Anchoring Proteins AKAP95 and HA95 (AKAp95/HA95), which is required to alter histone tail residues for aurora B kinase recognition at the onset of mitosis, is also impeded by HDIs. HDI treatment, by preventing aurora kinase B association with histone tails, therefore results in a delay in mitotic progression (Li, *et al*., 2006).

1.2.5.1. HDIs as cancer therapeutics

Strikingly, although both normal and tumour cells accumulate hyperacetylated histone residues, normal cells are ten times more resistant to HDIs than tumour cells (Dokmanovic and Marks, 2005; Minucci and Pelicci, 2006). This difference provides these agents with the powerful ability to specifically target cancer cells. The utility of

HDIs as anticancer agents is therefore not difficult to imagine, given that these drugs can induce cell-cycle arrest, apoptosis and terminal differentiation in neoplasms (Dokmanovic and Marks, 2005). Moreover, HDIs are particularly attractive as cancer therapeutics due to their anti-angiogenic, anti-metastatic and enhanced anti-tumour immunological qualities *in vivo* and *in vitro*.

HDIs suppress angiogenesis through the decreased expression of vascular endothelial growth factor (VEGF), HIF-1α and chemokine receptor 4 (CXCR4) as well as other pro-angiogenic genes (Kim, *et al*., 2001; Qian, *et al*., 2006). The down regulation of CXCR4 is of particular interest due to the role of CXCR4 in circulating endothelial cells to active sites of angiogenesis (Qian, *et al*., 2006). Moreover, the decreased accessibility in nutrient supply to a primary tumour in itself could be perceived as anti-metastatic. However, down regulation of matrix metalloproteinases (MMPs) or upregulation of MMP negative regulators, such as RECK (reversion-inducing cysteine-rich protein with Kazal motifs), by HDIs also inhibit metastasis. Importantly, an *in vivo* study involving hepatocarcinoma cell lines suggested that a modified HDI was able to specifically target the membrane protein CD44 and reduce the metastatic potential of these cell lines (Coradini, *et al*., 2004; Bolden, *et al*., 2006).

Enhancement of anti-tumour immunity by HDIs can be modulated through the alteration of immune cell activity and/or cytokine production. Indeed, HDIs are capable of making tumour cells more attractive immune targets. One way in which this occurs is through HDI -mediated induction of major histocompatibility complex (MHC) class I and II protein expression. Specifically, the expression of the MHC class I proteins MICA and MICB is induced in response to HDIs. These proteins are localized to the surface of tumour cells and are targets for the immunoreceptor of natural killer cell protein group 2D (NKG2D) found on the surface of natural killer and select T cells (Bolden, *et al*., 2006). The targeted binding of NKG2D to the tumour cell can result in cellular cytotoxicity, as demonstrated by HDI treated Hep3B and HepG2 hepatocarcinoma cell lines (Armeanu, *et al*., 2005; Bolden, *et al*., 2006).

Notably, HDIs can also act with other chemotherapeutic agents in a synergistic manner. Treatment of cells derived from both hematological and solid tumours with the DNA-demethylating agent 5-aza-cytidine followed by treatment with HDIs results in

the transcriptional activation of previously silenced genes (Bolden, *et al.*, 2006). The treatment of non-small-cell lung cancer cell lines with an HDI and anti-metabolite gencitabine resulted in synergistic cell death (Loprevite, *et al*., 2005; Bolden, *et al.,* 2006). Finally, the treatment of chronic myeloid leukemia (CML) cells with both HDIs and imatinib was effective against even imatinib-refractory CML (Nimmanapalli, *et al.*, 2003).

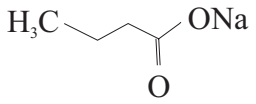
Significantly, HDAC overexpression is observed in many types of cancer. HDAC1 has been reported to be overexpressed in hormone refractory prostate, gastric, colon and breast cancers, as well as oesophageal squamous cell carcinomas. Similarly, HDAC2 overexpression is observed in colorectal, cervical and gastric cancers. Increased HDAC3 expression is seen in colon cancer and overexpression of HDAC6 has been observed in breast cancer. Furthermore, siRNA-mediated knockdowns of HDACs have resulted in suppressed cellular growth and survival (Glaser, *et al*., 2003; Wilson, *et al*., 2006). Taken together, these findings suggest an important role for HDACs in cancer and HDIs in cancer therapies.

1.2.5.2. HDI Families

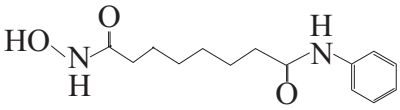
There are several families of HDIs grouped into classes based on their structure: (1) short-chain fatty acids (carboxylic acids), (2) Hydroxamates, (3) Benzamides, (4) Cyclic tetrapeptides, and (5) Electrophilic ketones (Bolden, *et al.*, 2006). Representative structures from each of the HDI families can be seen in Figure 1.3.

The first class of HDI is the short-chain fatty acid group; examples of this class include: sodium butyrate (SB) as well as the anti-epileptic valporic acid (VPA). The mechanism of action of these inhibitors is unknown. However, these agents are considered weak inhibitors of HDACs due to their short side chains, which limit their interaction with the tubular catalytic site of HDACs. As a result, the requirement of millimolar concentrations of this type of HDI makes this class a poor candidate for clinical development as a chemotherapeutic agent (Acharya, *et al.,* 2005). However, despite the relatively weak activity of these drugs several members of this class are currently undergoing clinical trials. VPA can inhibit all of the class I HDACs and HDAC4, 5, 7 and 9 and is implicated in the inhibition of angiogenesis and the induction of apoptosis in multi-drug resistant acute myeloid leukemia cells (AML). SB is a

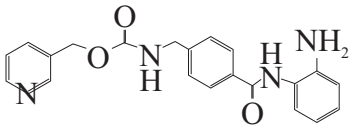
Short-chain fatty acids
(Sodium Butyrate)



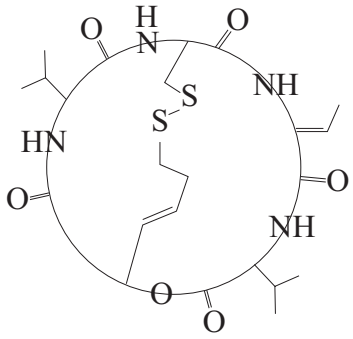
Hydroxamate
(SAHA)



Benzamide
(MS-275)



Cyclic tetrapeptide
(Depsipeptide)



Electrophilic ketone
(Trifluoromethylketone)

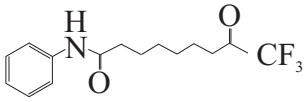


Figure 1.3. Structure of HDI family members. Representative examples of the structure of each class of HDI. The particular HDI depicted is given below the family member name in brackets.

naturally occurring HDI commonly present in colonic mucosa that selectively inhibits class I and II HDACs (Bolden, *et al.*, 2006). SB can cause cell cycle arrest and apoptosis in AML and acute T-lymphoblastic cells. The butanoic acid prodrug pivaloyl oxymethyl butyrate (AN-9) is, however, the most clinically promising short chain fatty acid HDI as it has been shown that AN-9 is ten-fold more potent than SB in leukemia tumour cell lines (Acharya, *et al*., 2005).

The hydroxamic acids are the broadest class of HDIs and have been studied most extensively. This class includes many potent HDIs in particular Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA) and Tubacin. This family is characterized as having a general structure consisting of a polar cap linked to a hydroxamic acid moiety via a bulky, hydrophobic linker region (Hess-Strump, 2005). The mechanism of inhibition by this class has been elucidated and the crystal stucture of HDAC8 interacting with TSA solved. The hydroxamic acid residues bind with high affinity to the tubular HDAC active site thereby preventing the access of the substrate to the enzymatically critical Zn^{2+} ion. The polar, bulky portion of the inhibitor acts as a cap to further discourage substrate-enzyme interaction (Somoza, *et al.*, 2004; Vannini, *et al.*, 2004). The majority of hydroxamic acid inhibitors are potent enough to function at nanomolar to micromolar concentrations *in vitro*. TSA was initially isolated as an anti-fungal agent (Bolden, *et al*., 2006). While, TSA is widely used in research it is unstable, relatively toxic and exhibits a lack of specificity towards particular HDACs, which makes it a poor candidate for clinical studies. However, TSA has been shown to be successful at inhibiting solid and hematological tumours in animal bearing models (Dukmanovic and Marks, 2005). Moreover, the potency of TSA has led to the creation of similar hydroxamic inhibitors that have demonstrated clinical success. SAHA is structurally similar to TSA and has activity at submicromolar concentrations. Although SAHA also inhibits all of the class I and class II HDACs, this HDI has been successful in phase I, II and III clinical trials and has been approved by the Federal Drug Administration (FDA) for use in the treatment of cutaneous T cell lymphomas (Bolden, *et al*., 2006; Marks and Breslow, 2007). The success of this particular hydroxamic acid is due to acceptable oral bioavailability, favourable pharmokinetic profile and the ability

to exert anti-cancer effects, at tolerable doses, in a wide variety of tumours (Dukmanovic and Marks, 2005). Tubacin is not currently undergoing clinical trials but is worth mentioning due its specificity towards HDAC6 activity. However, while Tubacin has been shown to elicit hyperacetylation of α-tubulin it has no effect on histone acetylation (Bolden, *et al*., 2006).

The benzamide class of HDIs is exemplified by two inhibitors: MS-275 and Cl-994. MS-275 is interesting due to the specificity this HDI exhibits towards HDAC1, 2, 3 although it has also been reported to marginally reduce HDAC8 activity. Thus this inhibitor has been reported to be specific only for class I HDACs and has entered phase II clinical trials (Bolden, *et al.*, 2006). CI-994, however, has greater clinical potential than MS-275 as it is currently in phase III clinical trials. Both of these inhibitors are functional at micromolar concentrations and have been successful in the treatment of hematological and solid tumours *in vivo* (Minucci and Pellicci, 2006; Bolden, *et al.*, 2006).

Depsipeptide is the most promising member of the cyclic tetrapeptides class of HDIs. Like MS-275, depsipeptide specifically targets class I HDACs and is currently in phase II clinical trials. Also, it is relatively potent as it is effective at nanomolar concentrations (Bolden, *et al.*, 2006). While the mechanism of action of this particular class of HDI is not known, it is hypothesized that the epoxyketone contained within the tetrapeptide may chemically modify the active site of the HDAC thus preventing enzyme-substrate formation (Acharya, *et al*., 2005). Depsipeptide can induce apoptosis in cells obtained from patients with chronic lymphocytic leukemia and has also demonstrated anti-angiogenic properties *in vivo* and *in vitro* (Kwon, *et al.*, 2002; Acharya, *et al*., 2005).

The electrophilic ketones are a small and relatively poorly defined class of HDIs. Trifluoromethylketone is functional at micromolar concentrations but HDAC specificity and clinical utility is currently unknown (Bolden, *et al*., 2006). Depudecin is an example from the miscellaneous class of HDIs and was originally isolated as a fungal metabolite. This particular HDI was identified due to the ability of depudecin to morphologically reverse transformed NIH 3T3 cells (Monneret, 2005). Subsequently, it

has been observed that depudecin hyperacetylates histones at micromolar concentrations by a currently unknown mechanism (Bolden, *et al*., 2006).

These diverse families of drugs are proving to be powerful chemotherapeutics. However, the mechanisms by which these drugs exert their effects remain largely unknown. While it is clear that apoptosis may be induced through the deregulation of genes leading either to the death receptor pathway or the mitochondrial pathway, it is not clear how these genes are deregulated. Similarly, it is known that HDIs stimulate G1 arrest through the transcriptional upregulation of $p21^{WAF1}$; however, the mechanism of transcriptional activation has not been satisfactorily explained. A great deal of study is still required to fully elucidate the mechanism of action of these pleiotropic drugs.

1.3. Eukaryotic transcriptional regulation

1.3.1. Requirements for basal transcription

Eukaryotic transcription is a complex process that begins with the sequential binding of essential factors to the DNA core promoter to ultimately produce mature messenger RNA (mRNA) transcripts designed to convert genetic information into the template for cellular protein synthesis. However, this simplified description of transcription fails to address the regulatory gauntlet required to produce a single peptide protein. The following sections will summarize pertinent checkpoints in mRNA maturation including promoter architecture and pre-initiation complex (PIC) assembly, promoter escape, transcriptional elongation and, finally, essential co-transcriptional processes and transcriptional termination.

1.3.1.1. RNA polymerase II -dependent transcription

Four discreet RNA Polymerases have been isolated and characterized. RNA Polymerase I is primarily involved in transcribing 18S and 28S ribosomal RNAs, while RNA Polymerase II transcribes mRNAs and RNA Polymerase III transcribes 5S ribosomal RNA, transfer RNAs and adenovirus VA RNAs. A fourth RNA Polymerase that appears to solely transcribe small interfering RNA (siRNA) was also recently identified in plants (Thomas and Chiang, 2006). The focus of this review will be the DNA-dependent RNA Polymerase II, as this polymerase is key in transcribing protein-encoding genes.

The eukaryotic core promoter is defined as the 40-50 bp region encompassing the transcriptional start site. Several *cis* acting elements have been identified at the core promoter region existing in variations of at least four basic forms: (1) the TATA-box, (2) Initiator element (Inr), (3) downstream activator element (DPE), and (4) TFIIB recognition element (BRE). These *cis* acting elements are the very first points of regulation for basal transcription as they are responsible for regulating the orientation of RNA Polymerase II and are required for transcriptional initiation (Smale and Kadonaga, 2003; Juven-Gershon, *et al*., 2006).

The TATA box was first identified in 1979 following a comparison of viral, *Drosophila* and mammalian RNA Polymerase II (RNA Pol. II) transcribed genes (Breathnach and Chambon, 1981). Although at one time the TATA-box was touted as

being almost ubiquitous at metazoan core promoter regions, it is now known that the TATA box is present at only ~24% of human promoter regions (Shi and Zhou, 2006). The characteristic TATA box consists of a TATA(A/T)AA(G/A) motif that appears -25 to -30 bp upstream of the transcriptional start site (Figure 1.4.) (Smale, 2001). This motif functions by acting as a point of recognition and docking site for the TATA-binding protein (TBP), which is necessary for transcriptional activation -mediated by RNA Pol. II. As TATA box sequence fidelity is directly related to TBP binding, the TATA box is involved in transcriptional regulation (Gross and Oelgeschlager, 2006). Although, the TATA box alone is sufficient for the recruitment of the PIC to the promoter region for basal transcription, the TATA box can co-exist with other core promoter elements, such as Inr elements, BREs and, occasionally, DPEs (Smale and Kadonaga, 2003).

Inr elements are relatively abundant core promoter elements reported to be present at ~46% of human genes. Significantly, the canonical (T/C)(T/C)AC(T/A)(T/C)(T/C) Inr elements exist at ~30% of human genes in the absence of a TATA box (Yang, *et al*. 2007). Clearly, these elements, which span the transcriptional start site (-2 to +4), do not require a TATA box to contribute to accurate transcription (Figure 1.4.). Despite the presence of these Inr elements at core promoters, little is understood concerning how these elements actually initiate transcription. While, it has been reported that the binding of TBP alone is insufficient to initiate transcription via this element, the binding of the general transcription factor (GTF) TFIID (transcription factor II D), which contains TBP, does initiate transcription. Significantly, two components of TFIID, TAF1 and TAF2 (TATA-binding protein associated factors 1, 2), have been suggested as being important in mediating TFIID binding to the Inr element; moreover, their involvement has been identified as being essential for Inr element promoter activity in both human and yeast cell extracts (Smale and Kadonaga, 2003). Furthermore, GTF TFIIA binding to the Inr element is essential for subsequent TFIID binding to this core promoter element although the binding site domain of TFIIA remains undetermined. Similarly, RNA Pol. II can itself recognize Inr elements, however, transcription initiated in the absence of other GTFs is inefficient (Smale and

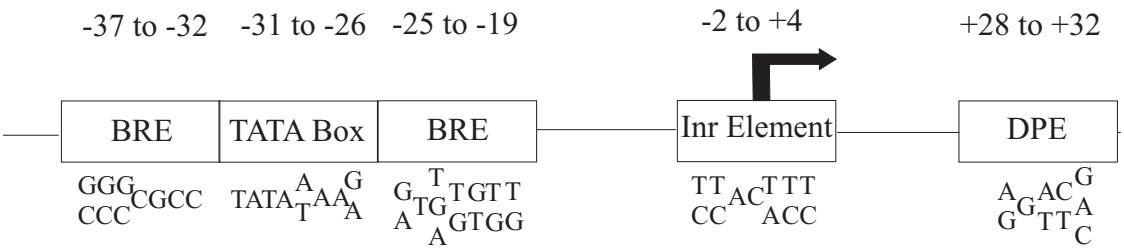


Figure 1.4. RNA Polymerase II core promoter elements. The four primary core promoter elements are depicted above. BRE stands for TFIIB response element, Inr element is the initiator element and DPE represents the downstream promoter element. Above each box is the location of the promoter element in reference to the transcriptional initiation start site (+1), which is signified by the arrow. Below each box contains the consensus sequence of each element represented.

Kadonaga, 2003). Taken together, these findings suggest that the Inr element requires the binding of all GTFs to successfully initiate transcription. Interestingly, Inr elements and TATA box elements can mediate synergistic transcriptional initiation whereby TFIID co-operatively binds both the TATA box and Inr element. However, this synergy is maintained only when the TATA box and Inr elements are within 30 bp of each other (Smale and Kadonaga, 2003; Juven-Gershon, *et al*., 2006). Inr elements and DPEs also act co-operatively to initiate transcription, although, in contrast to the TATA box, DPEs rely on Inr elements to mediate transcriptional initiation (Juven-Gershon, *et al*., 2006).

As the name suggests, downstream promoter elements (DPEs) are found +28 to +32 bp downstream of the Inr element and have the consensus sequence (A/G)G(A/T)(C/T)(G/A/C) that is conserved from yeast to humans (Figure 1.4.) (Smale and Kadonaga, 2003). This core promoter element is relatively under-represented in the human genome; it has been estimated that only ~12% of human core promoters contain this element (Jin, *et al*., 2006). Core promoters containing an Inr element and a DPE bind TFIID via both of these elements and, as stated above, DPEs require Inr elements to initiate transcription. Significantly, *in vitro* studies have demonstrated that a single nucleotide change in distance between the Inr element and DPE results in fold decreases in TFIID binding and dramatically decreases transcriptional initiation (Smale and Kadonaga, 2003).

In contrast to the TATA box, Inr elements or DPEs, BREs are located and are recognized by the GTF TFIIB whether upstream and/or downstream of the TATA box (-37 to -32 bp upstream and -25 to -19 bp downstream of the transcriptional start site). The upstream BRE, which mediates the interaction of TFIIB in the major groove upstream of the TATA box has a (G/C)(G/C)(G/C)CGCC recognition motif. The interaction of TFIIB with the minor groove downstream of the TATA box is, however, facilitated by the inexact motif (G/A)(T)(T/G/A)(T/G)(G/T)(T/G)(T/G) (Figure 1.4.) (Smale and Kadonaga, 2003). Interestingly, although BREs flank the TATA box, recent evidence suggests that a TATA box is not required for BRE -mediated TFIID binding. In fact, BREs may act as a substitute for the TATA box in transcriptional initiation (Deng and Roberts, 2006). Despite the presence of these elements in ~22% of human promoter regions the function of the BRE is poorly understood, as *in vitro* experiments

have described this promoter element as both increasing and decreasing transcriptional initiation (Smale and Kadonaga, 2003; Jin, *et al*., 2006).

Notably, not all core promoter regions contain the above mentioned core promoter elements. Generally, these promoter regions are CpG islands, which are composed of 0.5 Kb-2 Kb stretches of DNA possessing a relatively high density of CpG dinucleotides. It has been suggested that CpG islands account for nearly 50% of promoters of protein coding genes and generally, transcription is initiated via multiple transcription start sites that span ~100 bp or more (Smale and Kadonaga, 2003). Significantly, CpG islands and core promoter elements are not mutually exclusive.

1.3.1.2. Pre-initiation Complex Formation

As suggested in the previous section, transcriptional initiation -mediated by RNA Pol. II requires the co-operation of many essential components known collectively as general transcription factors (GTFs). The co-ordinated binding of GTFs and RNA Pol. II is referred to as the assembly of the pre-initiation complex (PIC) and is achieved through the stepwise and timely recruitment of all critical factors to the DNA core promoter. The GTFs governing PIC assembly include: transcription factor II A (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA Pol. II (Thomas and Chiang, 2006). Once the PIC has been assembled, the co-ordinated efforts of the PIC components initiate RNA Pol. II -mediated transcription (Figure 1.5.).

The TFIID is a large multi-protein complex consisting of TBP and at least 14 TATA-binding protein associated factors (TAFs) that include a wide range of molecular weights (250 KDa to 15 KDa) (Thomas and Chiang, 2006). As suggested above, human TFIID is capable of recognizing discreet core promoter elements such as the TATA-box, Inr element, and DPE *in vitro*. Upon binding these core promoter elements, TFIID can mediate the interaction between distal promoter elements and the general transcription machinery to promote PIC assembly. Also, TFIID acts to post-translationally modify chromatin and surrounding factors involved in the regulation of transcriptional initiation (Thomas and Chiang, 2006). An essential component of TFIID is TBP, which is responsible for TATA box recognition and binding. Upon binding the core promoter, crystal structures have demonstrated that the “saddle-like” TBP manipulates the promoter DNA by widening the minor groove and narrowing the major

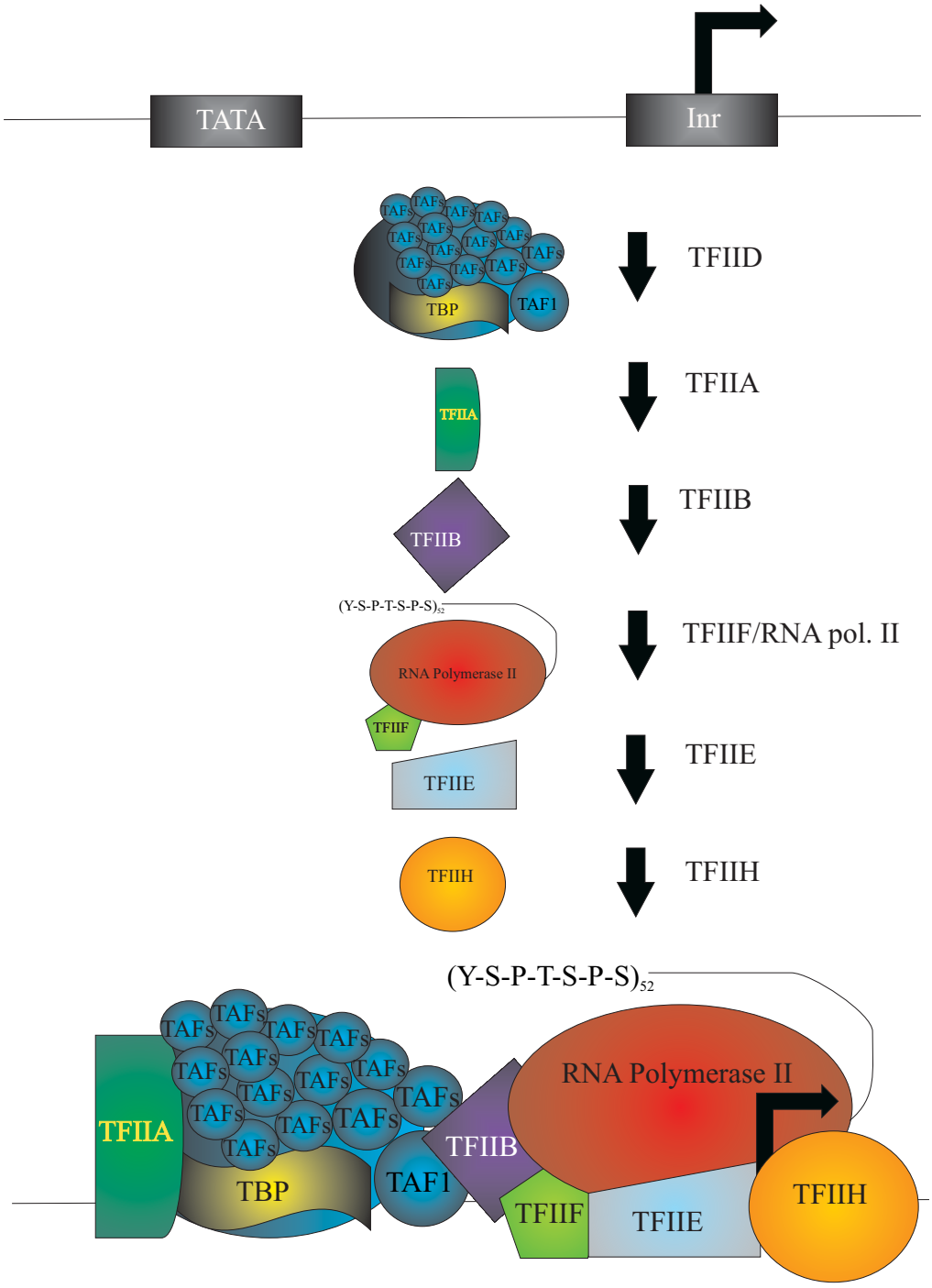


Figure 1.5. The assembly of the pre-initiation complex(PIC). The sequential assembly of general transcription factors (GTFs) on a core promoter containing both a TATA box and Inr element. Order of GTF recruitment to the promoter is indicated by arrows. TFIID is composed of TATA-binding protein associated factors (TAFs) and TATA-binding protein (TBP). PIC assembly results in basal transcription initiation (adapted from Roeder, 2005).

groove. The manipulation of the core promoter in such a way is important for the recruitment of the PIC (Thomas and Chiang, 2006). Similarly, the 250 KDa TAF1 and 150 KDa TAF2 components of TFIID play critical roles in promoter recognition via Inr elements, as well as in facilitating the effects of upstream activators on PIC assembly. TAF1 also modifies histone H1 by mono-ubiquitination and acetylates lysine residues on histone H2B/H3/H4 N-terminal tails, thus mediating structural chromatin changes required for transcriptional initiation. Furthermore, TAF1 contains two bromodomains that recognize acetylated histone H4K5 and H4K12 residues, as well as acetylated H3K9 and H3K14 residues, suggesting that TAF1 may aid in the recruitment of TFIID and chromatin remodelling enzymes to chromatin-packaged promoters (Green, 2000b; Wassarman and Sauer, 2001; Agalioti, *et al*., 2002).

TFIIA and TFIIB are the next PIC components to congregate at the core promoter following TFIID. Both TFIIA and TFIIB are reported to be involved in the stabilization of TBP binding to the TATA-box. Interestingly, work performed with a purified *in vitro* transcription system demonstrated that TFIIA is not strictly required for PIC assembly and initiation (Aoyagi and Wassarman, 2000). Conversely, TFIIB is absolutely required for TFIID/TBP core promoter stabilization, whether it be by stabilizing TATA box-TBP interactions or the manipulation of core promoter DNA through binding to both flanking BREs, TFIIB is essential for PIC assembly. Furthermore, the highly conserved TFIIB is also reported to have an essential role in the recruitment of RNA Pol. II/TFIIF to the partially assembled pre-initiation complex, as well as in transcriptional start site selection through the positioning of promoter DNA in the catalytic cleft of RNA Pol. II *in vivo* (Choi, *et al*., 2003; Thomas and Chiang, 2006).

TFIIF is responsible for the recruitment of RNA Pol. II to the partially assembled pre-initiation complex through pre-promoter binding interactions between TFIIF and RNA Pol. II (Svejstrup, 2003). Furthermore, TFIIF stabilizes the interactions between RNA Pol. II and promoter DNA by altering DNA topography such that the DNA is ensconced by RNA Pol. II and a stable TFIID-TFIIB-Pol. II-TFIIF complex is formed. The stabilization of RNA Pol. II by TFIIF aids in the prevention of spontaneous initiation by inhibiting the interaction between RNA Pol. II and non-promoter DNA sequences. TFIIF is also critical for the recruitment of both TFIIE and TFIIH, which are

responsible for facilitating RNA Pol. II promoter escape following transcriptional initiation and the enhancement of RNA Pol. II elongation (Thomas and Chiang, 2006).

RNA Pol. II is a 12 subunit, highly conserved, key enzymatic complex that facilitates every step of transcription. Structurally, human RNA pol. II is comprised of a N-terminal core domain that physically interacts with the promoter DNA, an 80-residue linker domain and a C- terminal domain (CTD) that is largely responsible for co-ordinating all phases of mRNA biogenesis. The human RNA Pol. II CTD is composed of 52 heptapeptide repeats of the consensus sequence Y1-S2-P3-T4-S5-P6-S7 (Meinhart, *et al* 2005). This heptapeptide is conserved across species; however, the number of repeats varies. In yeast, for example, a minimum of eight heptapeptide repeats are required for viability (West and Corden, 1995). The CTD is post-translationally modified at serine 2 (S2) and/or serine 5 (S5) by phosphorylation. Typically, while, the phosphorylation of CTD S5 is associated with the 5' region of the gene, CTD S2 phosphorylation is more common downstream of the promoter (Svejstrup, 2003). As a result of these findings, the phosporylation status of the CTD is often indicative of early or late stages of transcription (Komarnitski, *et al*. 2000). Upon entry into PIC assembly, however, the RNA pol. II CTD, in complex with TFIIF, is hypophosphorylated (Svejstrup, 2003; Thomas and Chiang, 2006). The modification status of the CTD plays an integral role in the formation of the CTD scaffold responsible for the binding of factors involved in initiation, elongation, mRNA 5' methyl-guanine capping, mRNA splicing, transcriptional termination and polyadenylation (Meinhart, *et al* 2005). In light of the numerous processes -dependent on the phosphorylation status of the CTD, the term "CTD code" has been coined (Buratowski, 2003) The N-terminal domain of RNA pol. II, based on crystal structures of RNA pol. II interaction with DNA, has been suggested to form a "jaw" that closes around the DNA upon RNA pol. II/TFIIF- DNA interaction. The N-terminal "jaw" domain is the RNA pol. II active site from which the nascent mRNA transcript extends upon transcription initiation. Importantly, it is at the RNA pol. II-DNA "jaw" interaction that TFIIE binds to the core promoter, 10 bp upstream of the transcriptional start site, to eventually be involved in mediating promoter melting and transcriptional initiation (Svejstrup, 2003; Thomas and Chiang, 2006).

As well as binding to RNA Pol. II, TFIIE physically interacts with TFIIF, TFIIB, promoter DNA and is responsible for the recruitment of TFIIH. The interaction between TFIIE and TFIIH is critical for initiation, as TFIIE is responsible for stimulating the DNA -dependent ATPase, CTD kinase and the DNA helicase activities of TFIIH. TFIIH is also associated with the cell cycle, as the CTD kinase domain of TFIIH is comprised of cyclin H and cyclin -dependent kinase 7 (CDK7), which is responsible for the phosphorylation of serine 5 within the CTD of RNA pol. II. (Svejstrup, 2003). TFIIH ATPase activity is required for stable promoter opening and the formation of the first phosphodiester bond, thereby signalling transcription initiation. Furthermore, without TFIIH, RNA Pol. II stalls at the promoter regions, which leads to abortive transcription products *in vitro*. Significantly, TFIIH is a bidirectional helicase, capable of unwinding DNA in both a 5'-3' and 3'-5' direction thus providing a transcription bubble comprised of single stranded promoter DNA that will act as the template for RNA Pol. II -mediated transcription (Svejstrup, 2003; Thomas and Chiang, 2006). Upon binding of TFIIH, the step-wise assembly of the PIC is complete (Figure 1.5.). Notably, it has been suggested that the PIC is not 'built" upon the core promoter elements, as described above, but rather binds to the core promoter elements as a pre-assembled holoenzyme. However, this theory has been mostly discounted as *in vitro* and *in vivo* studies have demonstrated that TFIID, TFIIA and TFIIB remain at the promoter region upon RNA Pol. II promoter clearance to usher in the assembly of a new PIC (Svejstrup, 2003).

1.3.1.3. RNA Polymerase II Promoter Escape

TFIIH is involved in transcriptional initiation as it phosphorylates serine 5 residues contained within the CTD of RNA Pol. II, mediates the formation of the first phosphodiester bond in mRNA biosynthesis, and further unwinds DNA downstream of the start site via helicase activity (Dvir, *et al.*, 2001). However, initiation does not guarantee the production of a mature mRNA transcript; the RNA Pol. II must "escape" from the core promoter region with the nascent mRNA transcript intact. This rate-limiting step is characterized by the formation of the first 8 nucleotides of mRNA and is mediated through the actions of TFIIB *in vivo* and *in vitro* (Saunders, *et al.*, 2006). Following the formation of a 4-5 nucleotide nascent mRNA transcript, the N-terminal

"B-finger" domain of TFIIB protrudes into the RNA Pol. II active site to stabilize the association of mRNA with RNA Pol. II (Chen and Hampsey, 2004; Saunders, *et al.*, 2006). The translocation of the RNA Pol. II active site to the ninth position after the start site marks the conversion from promoter escape into early elongation complex (EEC). The lengthening of the nascent mRNA transcript to 9 nucleotides also signifies the end of the TFIIH -mediated ATPase requirement for transcription and the collapse of the transcription bubble. Transcription bubble collapse also provides the required energy for the remodelling of the transcription complex. The EEC differs from the initiation complex in that it no longer contains TFIIA, TFIIB or TFIID and at least three proteins stabilize the nascent mRNA transcript to prevent DNA-RNA hybrid formation (Westover, *et al.*, 2004; Saunders, *et al.*, 2006).

1.3.1.4. Co-transcriptional Processes

Upon promoter escape, the EEC does not become a fully competent, mature elongation complex until after the transcription of ~30 nucleotides. At this stage, the EEC is paused or stalled on the DNA template by the binding of DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) to await the binding of pertinent factors to the RNA Pol. II S5 phosphorylated CTD. The capping enzyme (CE) and RNA (guanine-7) methyltranserase bind to the stalled RNA Pol. II S5 phosphorylated CTD to add a methylated guanosine "cap" to the 5'end of the RNA, thus protecting the nascent mRNA transcript from nuclease degradation (Sims III, *et al.*, 2004). After capping, positive transcription-elongation factor-b (P-TEFb) binds the CTD and phosphorylates DSIF, NELF, and S2 of the CTD, which relieves the negative pressure imposed by DSIF and NELF on elongation (Saunders, *et al*., 2006). Concurrently, S5 CTD phosphorylation is abrogated by the activity of the small CTD phosphates (SCPs) and TFIIS binds to the elongation complex to mediate the transition between promoter-proximal pausing and productive transcription elongation. Taken together, these findings highlight the importance of 5' mRNA capping on the production of a mature mRNA transcript. Without the interactions between the capping enzymes and P-TEFb, productive elongation would not occur and mRNA synthesis would be stalled 30 nucleotides into production. Furthermore, without a methyl-guanine cap nascent mRNAs may be degraded prior to being fully transcribed or exported into

the cytoplasm. Subsequent *in vitro* studies have identified components of the elongation complex to include: RNA pol. II, TFIIF, TFIIS, SCPs, P-TEFb, the elongation stimulating elongins and ELL (eleven-nineteen lysine-rich in leukemia), as well as Elongator and FACT (facilitates chromatin transcription), which are involved in facilitating transcription elongation in a chromatin environment (Sims III, *et al*., 2004).

Interestingly, the progression from the early elongation complex to productive elongation underscores the value of differential RNA Pol. II CTD phosphorylation. The deregulation of CTD phosphorylation stalls transcription initiation and elongation possibly due to aberrant enzymatic activity, be it through the loss of TFIIH kinase activity, the loss of SCP activity, or the loss of P-TEFb activity. Abnormal behaviour by any of these factors prohibits the production of a mature mRNA transcript.

5' capping is not the only RNA processing occurring in unison with initiation and/or elongation. mRNA splicing, -mediated by U1-U6 small nuclear RNAs (snRNAs), in association with transcription is essential for the production of a nuclear export-ready mature mRNA species. Recent reports suggest that snRNA U1 is a component of TFIIH and required for transcription initiation. Furthermore, transcription rates are reduced by the elimination of intronic sequences, while proximal pausing sites actually enhance transcription (Neugebauer, 2002). Similarly, 3' end formation and transcription have been linked through the observation that termination depends on the production of a transcript that includes a polyadenylation signal (AAUAAA), which can be as far as 1500 bp upstream of the termination site in humans. The polyadenylation of immature mRNA occurs in a region between the polyadenylation signal, which binds the cleavage and polyadenylation factor (CPSF), and a downstream G/U rich region reported to bind cleavage stimulatory factor (CstF). Cleavage factors I and II cut the mRNA and nuclear polyadenylation is achieved through the action of poly(A) polymerase (PAP) that binds to CPSF. Moreover, polyadenylation is considered a co-transcriptional process due to the findings that CPSF is bound to TFIID at the core promoter and the phosphorylated S2 CTD of RNA pol. II downstream of the core promoter. (Neugebauer, 2002; Orphanides and Reinberg, 2002).

1.3.1.5. Transcription through histones

In the previous sections, basal transcription from initiation to termination was described. The movement of the RNA Pol. II and associated complexes through a chromatin environment will now be discussed. *In vitro* studies with yeast cell free extracts have shown the ATP-dependent remodeller SWI/SNF to play a critical role in encouraging transcriptional initiation by altering the chromatin landscape at core promoter regions (Carey, *et al*., 2006). SWI/SNF works in concert with TFIIS to stimulate elongation after promoter-proximal pausing *in vivo* (Svejstrup, 2003). In yeast, the Paf1 complex associates with the S5 phosphorylated CTD to mediate the recruitment of methyltransferases required for histone modifications essential for transcriptional initiation, and is also implicated in elongation through the mediation of histone H3 and H4 eviction *in vitro* (Sims III, *et al*., 2004; Schwabish and Struhl, 2006). Alternatively, human Paf1 is associated with transcription dependent histone H3 acetylation, potentially providing the modifications necessary to attract the FACT complex to nucleosomes at transcriptionally active genes (Zhu, *et al*., 2005). As one of the components of the mature elongation complex, the FACT complex is intimately involved in facilitating transcriptional elongation through nucleosomes both *in vitro* and *in vivo*. Chromatin manipulation by FACT is achieved through nucleosomal disorder following the eviction of histone H2A-H2B dimers; furthermore, FACT histone chaperone activity reassembles the nucleosomes in the wake of RNA Pol. II (Workman, 2006). Although there is no evidence to support a physical interaction between FACT and RNA Pol. II, the FACT complex is consistently identified as being involved in the clearing of chromatin, thus allowing RNA Pol II transcription to proceed (Sims III, *et al*., 2004). The binding of FACT to histones may be associated with the elongation factor Elongator, the HAT activity of which may modify histone residues to recruit FACT to actively elongating transcripts. Interestingly, Elongator was first isolated as a component of the hyperphosphorylated form of RNA Pol. II. *In vitro* studies have demonstrated that Elongator is acetyl-CoA-dependent, further confirming an essential role for Elongator in either histone or protein acetylation (Svejstrup, 2003).

1.3.2. Proximal promoter sequences and activated transcription

The previous sections described the factors required for basal transcription. This section will integrate the effects of gene-specific upstream transcriptional regulators on activated transcription, as well as the factors and complexes mediating these effects.

1.3.2.1. Gene-specific proximal promoter elements

In yeast the region upstream of the DNA core promoter is known as the upstream activator sequence. In humans these gene-specific regulatory regions are frequently referred to as enhancers or proximal promoter sequences that are able to influence gene expression through the input of cellular cues such as growth, developmental and homeostatic signals (Malik and Roeder, 2005). The existence of these *cis* -acting components of transcriptional regulation in eukaryotes enables the rapid exchange of information to influence the genetic program in response to the cellular environment. There are numerous families of transcription factors that bind to cognate upstream promoter DNA sequences. However, the Sp family provides an excellent example of gene-specific transcription factors because they are frequently associated with the transcriptional regulation of housekeeping, tissue specific and inducible genes (Li, *et al.*, 2004).

The Sp family of transcription factors binds the *cis*-acting GC box DNA regulator elements containing the canonical 5'-GGGGCGGGG-3' sequence (Suske, *et al.*, 2005). There are at least nine Sp family members, although Sp1 and Sp3 are the only family members that are ubiquitously expressed. Sp1 is a powerful transactivator capable of synergistic activation of promoters containing multiple Sp binding sites. The synergistic activation by Sp1 is even more remarkable as Sp1 binding to sites as far away as 2 kb can be looped together to enhance Sp1-mediated transcriptional activation. Consistent with the role of Sp1 in activation, Sp1 is reported to recruit SWI/SNF chromatin-remodelling factors to promoter regions to increase the accessibility of promoter regions to other transcription factors (Li, *et al.*, 2004). Furthermore, Sp1 is capable of binding the GC box target sequence at promoter regions in assembled nucleosomes and further enhances transcription through CRSP (co-activator required for Sp1 activation) mediated RNA pol. II interaction *in vitro* (Ryu, *et al*., 1999; Suske, 1999).

Sp3 is similar to Sp1 in that Sp3 is able to mediate the activation of transcription. However, Sp3 can also act as an inhibitor of transcription. This phenomenon can be partially explained by the existence of four Sp3 translational isoforms. The two longest forms of Sp3 have only recently been characterized and are reported to function as activators. Previous studies had isolated a Sp3 isoform that was considered full length, although the N-terminal region present in the physiologically relevant forms of Sp3 was missing. Despite the absence of the N-terminal region, this putative long Sp3 isoform contained two glutamine rich activation domains and was also a transcriptional activator (Sapetschnig, *et al.*, 2004). The two shortest isoforms of Sp3, however, contain only one activation domain and, as a result, are frequently associated with transcriptional repression. Moreover, previous studies have suggested that these smaller isoforms could inhibit Sp1-mediated activation through competition for binding sites (Kennett, *et al.,*, 1997). As a result, the role exacted by Sp3 has been suggested to be defined by the sheer number of a particular Sp3 isoform present within the cell at a particular time (Li, *et al.*, 2004). Conversely, other groups have reported that the number of Sp binding sites within a particular promoter dictates the effect mediated by Sp3 in that system (Birnbaum, *et al*., 1995). Unlike Sp1, Sp3 does not bind to promoter regions as a multimer and as such is not capable of synergistic promoter activation. However, although Sp1 is involved in basal activation of the PKR gene (protein kinase regulated by RNA) Sp3 is the factor responsible for interferon-mediated inducible activation (Ward and Samuel, 2003; Li, *et al*, 2004). Subsequent experiments with Sp1 and Sp3 and this promoter system have suggested that Sp1-mediated basal activation of PKR was dominant over basal activation mediated by the longest Sp3 isoforms *in vivo* (Das, *et al.,* 2006). Clearly the activation and/or repression mediated by these factors are governed by mechanisms other than which factor is present in the greatest cellular abundance.

In order to rapidly alter gene expression, these factors must be amenable to a constantly shifting cellular environment. The post-translational modification of both Sp1 and Sp3 is an efficient mechanism to rapidly alter gene expression mediated by these factors. For example, in lung epithelial cells PP1-mediated dephosphorylation of Sp1 and Sp3 decreases the binding of these factors to the alpha-ENaC2 (alpha-epithelial

Na channel 2), thereby decreasing Sp1/Sp3-mediated activation at this promoter (Chu, *et al.*, 2003). However, it is likely that the effect mediated by a particular modification is dependent on the system affected. Indeed, *in vitro* studies using extracts isolated from human chronic mylogenous leukemia cells suggest that the phosphorylation of Sp1/Sp3 impedes the binding of these factors to the human Galpha(i2) gene promoter and thus reduces transcriptional activation mediated by these factors (Arinze and Kawai, 2003).

Similarly, both Sp1 and Sp3 are acetylated and this modification influences the ability of each of these factors to activate transcription. In a recent study it was suggested that the acetylation status of Sp1 determines which co-activators/co-repressors are recruited to the promoter of the 12(S)-lipoxygenase gene and therefore determines if the gene is repressed or activated (Hung, *et al*., 2006). Similarly, the acetylation of Sp3 is frequently associated with enhanced Sp3 -mediated activation (Ammanamanchi, *et al.*, 2003; Wooten and Ogretmen, 2006). However, Sp3 acetylation has also been shown to decrease Sp3-mediated activitation (Braun, *et al*, 2001). Sp3 acetylation is slightly more complicated than Sp1 acetylation, however, because the lysine residue within the inhibitory domain of Sp3 susceptible to acetylation can also be SUMOylated (Braun, *et al*, 2001; Ross, *et al*, 2002; Ammanamanchi, *et al.*, 2003; Splenger, *et al*, 2005; Ehlting, *et al*., 2006). Sp3 is SUMOylated via the protein inhibitor of activated STAT1 (PIAS) E3 ligase (Sapetschnig, *et al.*, 2002). Without exception, all reports of Sp3 SUMOylation are associated with decreased Sp3-mediated activation (Ross, *et al.*, 2002; Sapetschnig, *et al.*, 2002; Sapetschnig, *et al.,* 2004; Splenger, *et al.*, 2005). The mechanism of SUMO-1 -mediated inhibition of Sp3 activation is currently unknown. However, some suggest that SUMOylation alters the nuclear localization of Sp3 in murine cells, whereas, others claim that Sp3 nuclear localization is unaltered upon SUMO-1 modification (Ross, *et al.*, 2002; Sapetschnig, *et al.*, 2002). Moreover, transient transfection assays performed with *Drosophila* SL2 cells, which lack endogenous Sp factors, indicate that the SUMOylation of the shortest Sp3 isoforms was particularly crippling to the ability of these isoforms to activate the dihydrofolate reductase (DHFR) promoter (Splenger, *et al.*, 2005). Regardless of the mechanism, the modification of Sp3 by SUMO-1 remains a pertinent topic in transcriptional regulation by gene-specific transcription factors.

1.3.2.2. Activated Transcription

Gene-specific transcriptional regulators bind regions upstream of the core promoter. In order for these factors to influence the assembly of the PIC and the recruitment of other factors required for transcription, therefore, co-activators and co-repressors are required to communicate the effects imposed by the binding of gene-specific transcription factors to the core promoter. The Mediator complex is one of the best-characterized examples of a co-activator, or co-repressor, required for activated transcription in metazoans (Roeder, 2005).

Studies performed in yeast have characterized the yeast Mediator as being composed of ~20 subunits that form four distinct Mediator subdomains known as the "head", "middle", "tail" and kinase modules (Conaway, *et al*., 2005). Functional characterization has demonstrated that yeast Mediator is important in activator-dependent transcription as Mediator associates with both the transcriptional activation domain of gene-specific transcription factors and also with the hypophosphorylated form of the RNA Pol. II CTD. However, Mediator is not just an adapter protein complex linking upstream transcription factors and PIC components. *In vitro* transcription assays indicate that the binding of yeast Mediator to RNA Pol. II stimulated the CTD-kinase activity of TFIIH, resulting in the phosphorylation of S5 on the CTD, which is critical in initiation (Conaway, *et al*., 2005). Moreover, the Mediator/RNA Pol. II interaction may be important only at initiation because Mediator is absent from elongator complexes containing hyperphosphorylated forms of the RNA Pol. II CTD, thus suggesting that CTD phosphorylation may disrupt Mediator binding (Svejstrup, 2003). Furthermore, yeast Mediator can also function as a HAT thereby providing a connection between chromatin remodelling and activated transcription (Conaway, *et al*., 2005). Taken together, these results indicate that yeast Mediator has essential functions at many levels of activated transcription.

The human Mediator complex was first isolated through association with the liganded thyroid receptor (TR) and was named TRAP for thyroid hormone receptor-associated proteins. The TRAP complex contained subunits that were homologous to yeast Mediator subunits and was shown to be required for TR-dependent transcription from DNA templates by RNA Pol. II (Roeder, 2005). Subsequent isolation of other

human Mediator complexes have identified at least 30 subunits that contribute to activated transcription in human cells *in vitro* and have identified a role for discreet Mediator (MED) subunits in directing transcription in cooperation with specific activators both *in vivo* and *in vitro* (Malik and Roeder, 2005; Zhang, *et al*., 2005). As observed with yeast cell free extracts, human MED subunits associate with the hypophosphorylated CTD of RNA pol. II *in vitro* (Näär, *et al*., 2002). While the functional consequences of this interaction have not yet been delineated, *in vivo* ChIP studies have indicated that during hormone-induced target gene activation MED subunits are recruited to the core promoter region before the recruitment of RNA Pol. II (Sharma and Fondell, 2002; Malik and Roeder, 2005). These studies suggest that, like yeast Mediator, human Mediator may have a role in the formation of the PIC at the promoters of activated genes.

Interestingly, many human co-activator complexes contain a heterogeneous assortment of MED subunits, which may be involved in dictating what role Mediator imparts on activated transcription. For example, the Sp1 associated CRSP complex contains MED components with homology to the "head", "middle" and "tail" regions of the yeast MED. However, it has been reported that the CRSP complex lacks the MED subunits responsible for Mediator kinase activity that are required for Mediator facilitated co-repression in yeast. *In vitro* transcription studies using both naked DNA and chromatin templates have also identified a role for CRSP in activator -dependent transcription activation. Conversely, the much larger TRAP Mediator complex contains components of all four modules and is involved in both the stimulation and inhibition of activator-dependent transcription *in vitro,* suggesting that the kinase module may be required for co-repressor MED activity (Conaway, *et al*., 2005). As a result, it has been suggested that the repressive nature of the kinase module may be the result of interference in MED/ RNA Pol. II binding, and/or due to the phosphorylation and resulting deactivation of TFIIH by the MED kinase module (Malik and Roeder, 2005). Presumably, the alternative composition of human MED containing complexes may be explained by the interaction of specific MED subunits with the activation domains of different gene-specific transcriptional activators to elicit alternative regulatory programs (Conaway, *et al*., 2005). Taken together, human and yeast Mediator studies have

suggested a requirement for MED subunit containing complexes in exerting the effects of gene-specific transcription factors. Furthermore, the high degree of evolutionary conservation existing between human and yeast Mediator subunits suggests that human Mediator may prove to be functionally equivalent to its yeast counterpart.

As described in the previous sections, transcriptional regulation of mammalian genes requires the input of a variety of diverse factors. Basal transcription requires the interplay of core promoter sequences, GTFs and RNA Polymerase II. However, gene-specific transcriptional activation involves the inter-relation of diverse cellular signals manifested through the modifications of gene-specific transcription factors, co-activators and co-repressors, such as Mediator, and all of the components of the basal transcription apparatus. The high level of regulation of these interactions has been developed to co-ordinate the adaptive nature of the mammalian genetic program.

1.4. c-Src

1.4.1. Src

Nearly a century ago Peyton Rous first suggested that a chicken tumour could be transfered to another chicken of the same species using cell-free filtrates or a "filterable agent" (Rous, 1911). At this time it was not known that "filterable agents", or viruses as we know them now, contained genetic information. Continued work by Rous and that of at least two other groups in-dependently demonstrated that a virus could indeed induce tumours in healthy specimens. Despite considerable resistance from the scientific community, this controversial finding was eventually rewarded when Peyton Rous received the Nobel Prize in 1966 (Martin, 2004).

Since these initial findings, a wealth of information concerning the Rous sarcoma virus (RSV) has emerged. In 1955, a landmark study pioneered by Harry Rubin demonstrated that every cell in a RSV-induced tumour was capable of disseminating the virus thus suggesting that the virus had become a permanent component of the tumour and was actually necessary to maintain the tumour's malignant state (Rubin, 1955). In the 1960s, several laboratories in-dependently identified that transforming and replication qualities could be separated in a particular strain of RSV. These findings, and those of others, led to the observation that the RNA genome of transforming, replication positive RSV strains were larger than their non-transforming counterparts. The transforming strain of RSV was identified as containing unique oligonucleotides, through the use of oligonucleotide fingerprinting and chromatography, in this manner viral Src (v-Src) was identified (Wang, *et al*., 1976). Concurrently, the concept that viral genomes might contain captured cellular genes was gaining ground, and the findings that transformation negative strains of RSV could still replicate suggested that the mutated transforming gene (v-Src) from the RSV might actually be a mutated endogenous gene (Martin, 2004). A v-Src specific probe was annealed to normal avian DNA and a cellular component for v-SRC, aptly named cellular Src (c-Src), was identified (Stehelin, *et al*., 1976). Moreover, the cDNA probe was also able to bind higher vertebrate DNA, thus confirming the existence of a cellular component to v-Src that is conserved through evolution. v-Src had already been dubbed an oncogene due to its ability to transform normal cells. As such, the term proto-oncogene was introduced

to distinguish between the transforming v-Src gene and the c-Src gene that is non-transforming unless overexpressed and/or mutated (Martin, 2004). Src has been implicated in tumourgenesis in one capacity or another for nearly a century. It is therefore not surprising that the roles of c-Src in tumour formation continue to be a popular topic in tumour biology. The structure and function of Src are essential to understanding the role of Src in the oncogenic process.

1.4.2. c-Src Protein Structure and Regulation

Studies initially performed with v-Src identified Src as a 60 kDa, non-receptor tyrosine kinase. Interestingly, it was originally thought that v-Src was a threonine kinase as tyrosine kinases were unheard of at that time. However, in 1980 a group was able to prove that not only did v-Src specifically phosphorylate tyrosine residues but that v-Src itself was tyrosine phosphorylated (Hunter and Sefton, 1980). These observations hinted at a role for phosphorylation in Src activity and function. v-Src autophosphorylation was subsequently shown to occur at tyrosine residue 416 (Tyr416) (Tyr419 in humans). However, while this site in c-Src was not found to be phosphorylated *in vivo*, tyrosine residue 527 (Tyr530 in humans), a residue that is absent in v-Src, was phosphorylated, furthermore, the phosphorylation of c-Src at Tyr527 was shown to be repressive to c-Src tyrosine kinase activity, while dephosphorylation activated c-Src kinase. Significantly, Tyr527 phosphorylation of c-Src was found to be due to the action of Csk (carboxy-terminal Src kinase), rather than a product of auto-phosphorylation (Martin, 2001). Eventually it was ascertained that c-Src was also autophosphorylated at Tyr 416; however, dephosphorylation of Tyr527 was a prerequisite to Tyr 416 autophosphorylation (Parsons and Weber, 1989). These results suggested that v-Src was a constitutively activated form of c-Src, as the negative regulatory C-terminal was not present in v-Src. Already it was clear that structural differences between v-Src and c-Src were involved in mediating their alternate effects.

In the course of delineating c-Src structure, the existence of cellular homologues bearing significant similarity to c-Src were described and given the name of Src family kinases (SFKs). Currently there are nine members in this structurally related family of non-receptor tyrosine kinases; however, while structurally similar, expression patterns

differ dramatically between family members (Lowell and Soriano, 1996). For example, Fyn and Yes are the two SFKs most closely related to Src and, like Src, demonstrate an almost ubiquitous distribution. Conversely, the other SFKs are more tissue specific and as a consequence their expression is primarily restricted to haematopoietic and neuronal cells (Lowell and Soriano, 1996; Martin, 2001).

All of the Src family members share a similar structure consisting of six discreet functional domains (Figure 1.6.). These domains include: a short regulatory C-terminal tail domain, the 250-residue Src homology 1 (SH1) catalytic domain, the 100-residue SH2 domain, the 50-residue SH3 domain, the 40-70-residue unique region and finally, the 15-residue SH4 domain (Thomas and Brugge, 1997). The regulatory C-terminal domain contains the hallmark Tyr530 residue common to all SFK. As described earlier, the phosphorylation of this residue by Csk has a negative impact on Src kinase activity (Sicheri and Kuriyan, 1997). The catalytic SH1 domain contains Tyr419, which is autophosphorylated to activate Src. The SH2 domain binds phosphotyrosine; however, binding specificity is determined by the presence of a second binding pocket. This second binding pocket recognizes residues immediately C-terminal to the phosphotyrosine residue. *In vitro* experiments have suggested that the phosphopeptides optimal for binding contain a YEEI motif (Y = tyrosine, E = glutamic acid, I = isoleucine) (Pawson, 2004). The SH3 domain interacts specifically with proline-rich sequences containing a PXXP (P = proline, X = any amino acid residue) consensus sequence to form a left-handed polyproline helix (polyproline type II) (Li, 2005). As the name suggests, the unique domain is a region poorly conserved between Src family members. This region is responsible for interactions between discreet Src family members and effectors (Sicheri and Kuriyan, 1997). Finally, the SH4 domain is required for cotranslational attachment of myristic acid by the action of *N*-myristoyl transferase (NMT). Myristylation of this domain is key in Src function as it results in the localization of c-Src to membranes including: the rough endoplasmic reticulum, endosomes, secretory vesicles and the plasma membrane (Silverman and Resh, 1992). Other SFK members are also modified at the N-terminal region, however, palmitic acid is more commonly the lipid modifier (Lowell and Soriano, 1996; Thomas and Brugge, 1997).

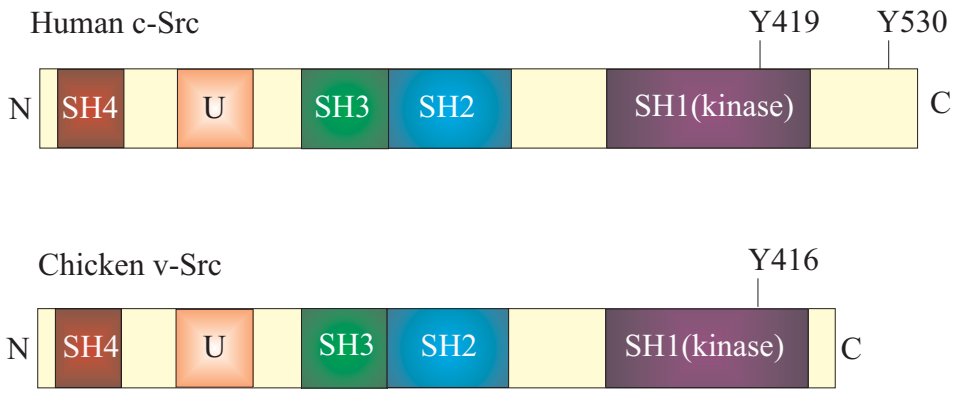


Figure 1.6. Structure of Src proteins. Comparison of protein structures of human c-Src and chicken v-Src. Both proteins contain four Src homology domains (SH). SH1 is the catalytic domain which contains the conserved tyrosine residue required for autophosphorylation and activation (Tyr419 in human c-Src and Tyr416 in chicken v-Src). The SH2 domain is required for the recognition of phosphotyrosine residues. The SH3 domain binds proline-rich sequences. The unique domain is a region exhibiting very little conservation between Src family members. The SH4 domain is required for myristylation which aids in the localization of Src to cellular membranes. c-Src and v-Src differ primarily at the C-terminal domain. v-Src lacks the negative regulatory Tyr530 residue which allows v-Src to be constitutively activated (Yeatman, 2004).

Src structure is critical in understanding c-Src regulation. Crystallographic and structure-function studies have confirmed that Src is regulated through interactions involving the SH2, SH3 and C-terminal domains (Frame, 2001). In particular, as mentioned previously, Tyr530 within the C-terminal domain is phosphorylated by Csk (Martin, 2001). The resulting phosphotyrosine and associated residues interact with the SH2 domain to result in a closed or "inactive" conformation. Subsequently, or concurrently, the proximity between SH3 and the region linking the kinase and SH2 domains increases to allow the binding of SH3 to proline rich sequences within the linker region. These interactions further confine c-Src and render the Tyr419 residue within the kinase domain incapable of autophosphorylation thus hindering c-Src kinase activity (Figure 1.7.). The preclusion of c-Src autophosphorylation is not the only interaction preventing kinase activity. The interactions between the SH2 and the C-terminal domain, as well as SH3 and the linker region, prohibit associations between these domains and other proteins, including receptor tyrosine kinases (Frame, 2001; Yeatman, 2004). Appropriately, the dephosphorylation of Tyr530 relieves the "closed", inactive c-Src conformation to again allow the autophosphorylation of Tyr419 and intramolecular interactions between the SH2/SH3 domains with downstream targets such as focal-adhesion kinase (FAK) (Frame, 2001). Dephosphorylation of Tyr530 is accomplished through the activities of phosphatases such as: Protein Tyrosine Phosphatase-α (PTP-α), PTP1, PTP2, SH2 containing phosphatase 1 (SHP1) and SHP2 (Yeatman, 2004).

1.4.3. c-Src Signalling

c-Src activation is associated with numerous cellular processes -mediated through interactions with proteins and signalling molecules. Two of the most commonly studied c-Src interactions will be briefly reviewed: the interaction with members of the epidermal growth factor (EGF) family of receptors to mediate cellular proliferation and the association with focal adhesion kinase (FAK) to mediate cellular migration, motility and invasion.

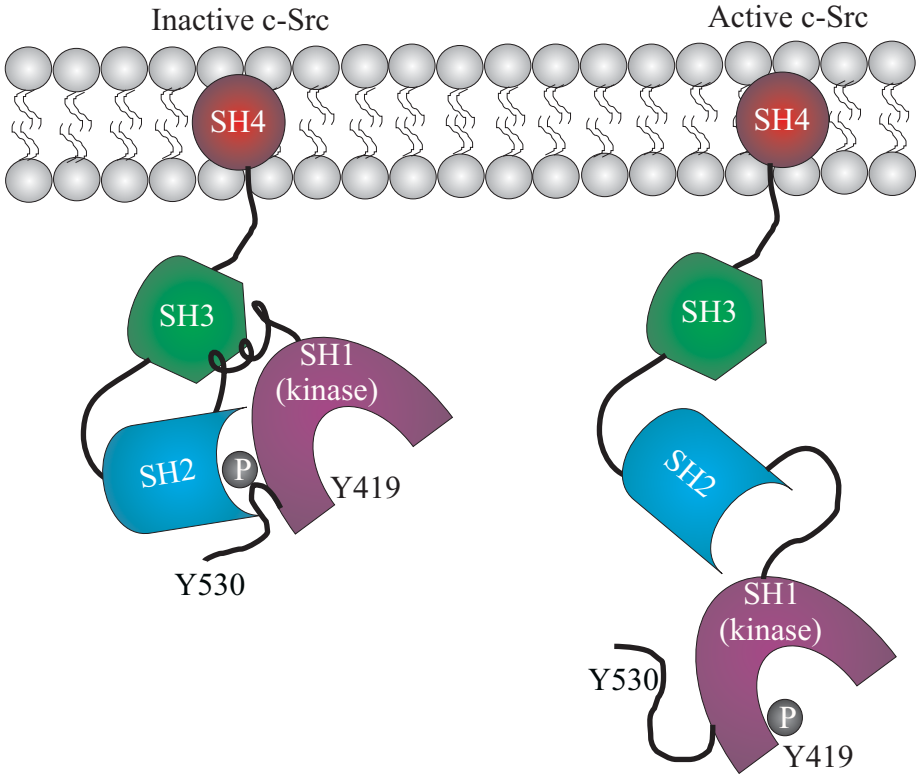


Figure 4.7. c-Src activation. Csk phophorylates Y530 resulting in an interaction between phosphotyrosine 530 and the SH2 domain. Concurrently, SH3 interacts with the linker region of Src thus closing c-Src off from interactions with RTKs and resulting in an inactive, closed enzymatic conformation. Dephosphorylation of Y530 relieves the closed, inactive conformation of c-Src to allow for the autophosphorylation of Y419 and SH2/SH3 domains the opportunity to interact with downstream targets (Yeatman, 2004).

The interactions between c-Src and transmembrane receptor tyrosine kinases (RTKs) are well documented and are fundamental in mediating c-Src activity (Parsons and Parsons, 1997). The effects of these interactions involve the autophosphorylation of c-Src, the association of c-Src and RTKs to mediate downstream events, and the phosphorylation of the RTK itself (Thomas and Brugge, 1997). The interactions between c-Src and EGFR characterize the properties of c-Src and RTK associations and provide excellent examples of the processes pivotal to c-Src participation. Pioneering studies in murine cells have demonstrated that c-Src physically associates with activated EGFR via the SH2 domain, which prompts the transient activation of c-Src through the auto-phosphorylation of Tyr419 and results in the phosphorylation of downstream targets. Similarly, c-Src overexpression in murine fibroblasts enhances the phosphorylation of EGFR downstream effectors such as p190 Rho-Gap (p190 Rho GTPase activating proteins). Taken together, these experiments suggest that c-Src and EGFR function in a synergistic manner. Moreover, the overexpression of c-Src in quiescent murine cells enhances DNA synthesis in response to EGF, thereby suggesting a role for c-Src in EGFR -mediated G_0 to G_1 cell cycle progression (Parsons and Parsons, 1997). More recent experiments have suggested that EGFR is itself a target for c-Src kinase activity at multiple residues. In particular, c-Src directly or indirectly mediates Tyr845 phosphorylation of EGFR, which is required for cyclooxegenase (Cox) II -mediated enhanced cell survival and EGF-induced cell proliferation through STAT5b. c-Src also negatively regulates EGFR degradation by facilitating the ubiquitination and proteosomal degradation of CBL (Casitas B-lineage Lymphoma), an E3 ligase that promotes receptor endocytosis and degradation (Ishizawar and Parsons, 2004). Interestingly, c-Src is itself a target for CBL proteosomal degradation which suppresses v-Src transformation in NIH 3T3 cells (Kim, *et al*., 2004).

FAK and c-Src transiently associate following ligand stimulation of the EGF receptors (see previous section) or integrin/extracellular matrix (ECM) complex formation. This association potentiates the autophosphorylation of FAK, which results in FAK engagement with a variety of downstream effectors, including c-Src. FAK/c-Src association activates c-Src, consequently causing the phosphorylation of tyrosine residues to enhance FAK kinase activity and provide the docking sites required for

interactions with other signalling molecules (Ishizawar and Parsons, 2004). The significance of this interaction has consequences to cellular adhesion, motility and invasion through the destabilization of focal adhesions and adheren junctions. For example, focal adhesions are formed at the sites where the actin cytoskeleton is linked to the ECM by integrins. Focal adhesions are highly dynamic and can consist of over 50 different proteins, including both c-Src and FAK, which are involved in mediating both cell-matrix attachment and cell-matrix detachment to induce cellular migration and motility. c-Src and FAK are primarily involved in detachment through the disruption of focal adhesions and actin stress fibres, which results in cellular migration. Specifically, c-Src -mediated activation of FAK causes the phosphorylation of downstream targets including integrin associated CAS (CRK-associated substrate) and the integrin-actin cytoskeleton connector paxillin to alter the cytoskeleton to promote focal-adhesion turnover and allow motility (Yeatman, 2004). Significantly, *Fak*$^{-/-}$ cells transfected with dominant-negative c-Src display larger than average focal adhesions and reduced motility, further confirming the value of c-Src-FAK interactions in cellular migration (Ilic, *et al*., 1995). Moreover, c-Src activated FAK stimulates the JNK (c-JUN amino terminal kinase) pathway to ultimately result in the increased expression of MMP2/MMP9, which promote the breakdown of the ECM. The disintegration of the ECM is required for the invasion of tumour cells to surrounding tissues, thereby directly linking c-Src/FAK interactions to tumour progression (Yeatman, 2004).

1.4.4. c-Src and Cancer

The previous section described only a few cellular processes involving c-Src. Given the breadth of critical interactions requiring c-Src and the effects -mediated by these interactions, including proliferation, cellular migration, motility and invasiveness, it comes as no surprise that the constitutively activated v-Src is an oncogene. However, c-Src activation and/or overexpression have also been implicated in tumour progression.

In-dependent groups have reported the presence of an activating mutation of c-Src in ~12% of advanced colon cancers and very small portion of endometrial carcinomas (less than 2%) (Irby, *et al*., 1999; Sugimura, *et al*., 2000). This activating mutation results from the premature truncation of the c-Src C-terminal domain at

glutamine residue 531, which prevents the negative regulation of c-Src by phosphorylation at Tyr530. Moreover this change was demonstrated to be activating, transforming, tumourgenic and metastasis promoting (Irby, *et al*., 1999). Subsequent studies with colon cancer patients have not been able to confirm the presence of this activating mutation in advanced colon carcinomas; as such it has been suggested that these mutations may be present in only a very small subset of colon cancers (Daigo, *et al*., 1999; Nilbert and Fernebro, 2000). Significantly, these activating mutations are not the only studies reporting a correlation between increased c-Src kinase activity and cancer.

Several groups have reported increased c-Src expression and activity in colonic polyps and adenomas as compared to normal mucosa (Talamonti, *et al*., 1993; Iravani, *et al*., 1998). Specifically, it appears that both c-Src expression and c-Src activity are increasing during adenoma formation, whereas only c-Src activity continues to increase as the cells become tumourous and possibly metastatic (Yeatman, 2004). Similarly, high c-Src kinase activity has been correlated with a poor clinical prognosis in all stages of human colon carcinomas (Aligayer, *et al*., 2002). To further examine the role of c-Src activity in colon cancer cells, several groups have studied the effects of downregulating c-Src activity on cellular phenotypes. In one such study, Csk or a dominant-negative form of c-Src was overexpressed in a rat carcinoma cell line. Although these cells were still able to form primary tumours, they could no longer metastasize to secondary sites when injected into rats (Boyer, *et al*., 2002). Along a similar vein, a c-Src anti-sense cell line was developed using the colon cancer cell line, HT29. These cells grew far more slowly than their parental counterparts, further suggesting a role for c-Src in proliferation. Furthermore, when these anti-sense cells were injected into nude mice, the anti-sense producing cells formed tumours at a greatly reduced rate as compared to wild type counterparts (Staley, *et al*., 1997). The mechanisms behind these effects are complex and likely involve the co-ordination of many signalling pathways.

As reviewed in the previous section, Src and EGFR activity are intimately related and their association results in synergistic activation of downstream targets that encourage cellular proliferation. To underscore the importance of this interaction in regard to cancer, c-Src and members of the EGFR family are co-overexpressed in 70%

of breast cancer tumours (Ishizawar and Parsons, 2004). Increased c-Src activity, mediated by RTKs, results in the STAT3 -mediated induction of VEGF (Yeatman, 2004; Yu and Jove, 2004). Significantly, c-Src is required for hypoxia-induced VEGF expression to mediate angiogenesis *in vivo* (Kilarski, *et al*., 2003). Furthermore, through the use of *in vivo* models of angiogenesis it has been shown that Src inhibition is an effective mechanism in blocking neovascularization, thus confirming an angiogenic role for Src (Summy and Gallick, 2006).

The interactions between FAK and c-Src also deserve attention with respect to cancer progression. These two kinases interact to mediate cellular migration, motility and invasion. The assembly and disassembly of focal adhesions, mediated by Src and FAK, is a normal cellular occurrence during mitosis and normal cellular migration. However, focal adhesion disassembly also occurs during transformation to allow for increased migration (Yeatman, 2004). This provides another example of how a normal cellular process can become potentially tumourgenic if enhanced through the heightened activity of a key kinase, such as c-Src.

Increased c-Src activity can be achieved through a variety of mechanisms. As described earlier, an activating mutation is able to convert c-Src into a potent oncoprotein. However, this particular mutation appears to exist only in a very small subset of tumour samples. Another way in which an increase in c-Src activity may occur is through the downregulation of Csk levels, which would result in decreased phosphorylation of the C-terminal Tyr residue of c-Src. This is the case in both colon cancer carcinomas and hepatocellular cancers, in which Csk is underexpressed and c-Src is overexpressed, resulting in higher levels of c-Src activated protein (Yeatman, 2004). Significantly, Src overexpression achieved through transcriptional activation also results in increased c-Src protein levels accompanied by elevated kinase activity in a variety of colon cancer cell lines (Dehm, *et al*., 2001). However, future studies into the mechanism of c-Src deregulation will be necessary to fully elucidate the role of Src in cancer.

1.5. SRC Transcriptional Regulation

The SRC gene consists of 15 exons spanning over 60 Kb (Figure 1.8.) (Bonham and Fujita, 1993; Bonham, *et al*., 2000). Exons 2-12 encode the open reading frame and 3' untranslated region (3'UTR). Exons 1B and 1C encode a portion of the 5'UTR while the remainder of the 5'UTR and the alternative SRC promoter regions are encoded by exon 1α and exon 1A. The SRC1α promoter is ~1 Kb upstream of SRC1A promoter, however, transcripts initiated from either promoter are alternatively spliced to exon 1B to yield mRNA products with differing 5'UTRs that encode identical protein products (Figure 1.8.). Interestingly, relative SRC promoter usage was measured in several colon cancer and hepatocellular carcinoma cell lines, and while the SRC1A and SRC1α promoters each contribute equally to mRNA transcribed in the colon cancer cell lines HT29, COLO 201 and COLO205, in the hepatocellular carcinoma HepG2 cells a greater number of transcripts were initiated from the SRC1α promoter (Bonham, *et al*., 2000).

1.5.1. SRC1A Promoter

The SRC1A promoter is a housekeeping-like promoter; it is GC rich, ubiquitously expressed and transcription is initiated via an Inr element (Bonham and Fujita, 1993; Dehm, *et al*., 2004). This well characterized promoter is regulated by two critical Sp1/Sp3 binding sites (GC1 and GA2) and three perfect polypurine/polypyrimidine tracts that bind heterogeneous nuclear ribonucleoprotein K (hnRNP K) (TC tracts 1, 2 and 3) (Ritchie, *et al.*, 2000; Ritchie, *et al*., 2003).
The Sp family of transcription factors has been reviewed in a previous section but the interactions between SRC1A and Sp family of transcription factors will be described here. *In vitro* experiments have demonstrated that both Sp1 and at least two forms of Sp3 (a longer and shorter form) bind to the GC1 and GA2 sites upstream of the SRC1A core promoter region. Furthermore, initial transient transfection experiments indicated that Sp1 is a strong activator of SRC1A activity whereas Sp3 has little effect on SRC1A activation in the Sp factor deficient *Drosophila* SL2 cell line. Upon co-transfection with both Sp1 and Sp3 factors, Sp3 was able to impede Sp1-mediated activation, presumably through competition for binding sites (Ritchie, *et al.*, 2000). To further

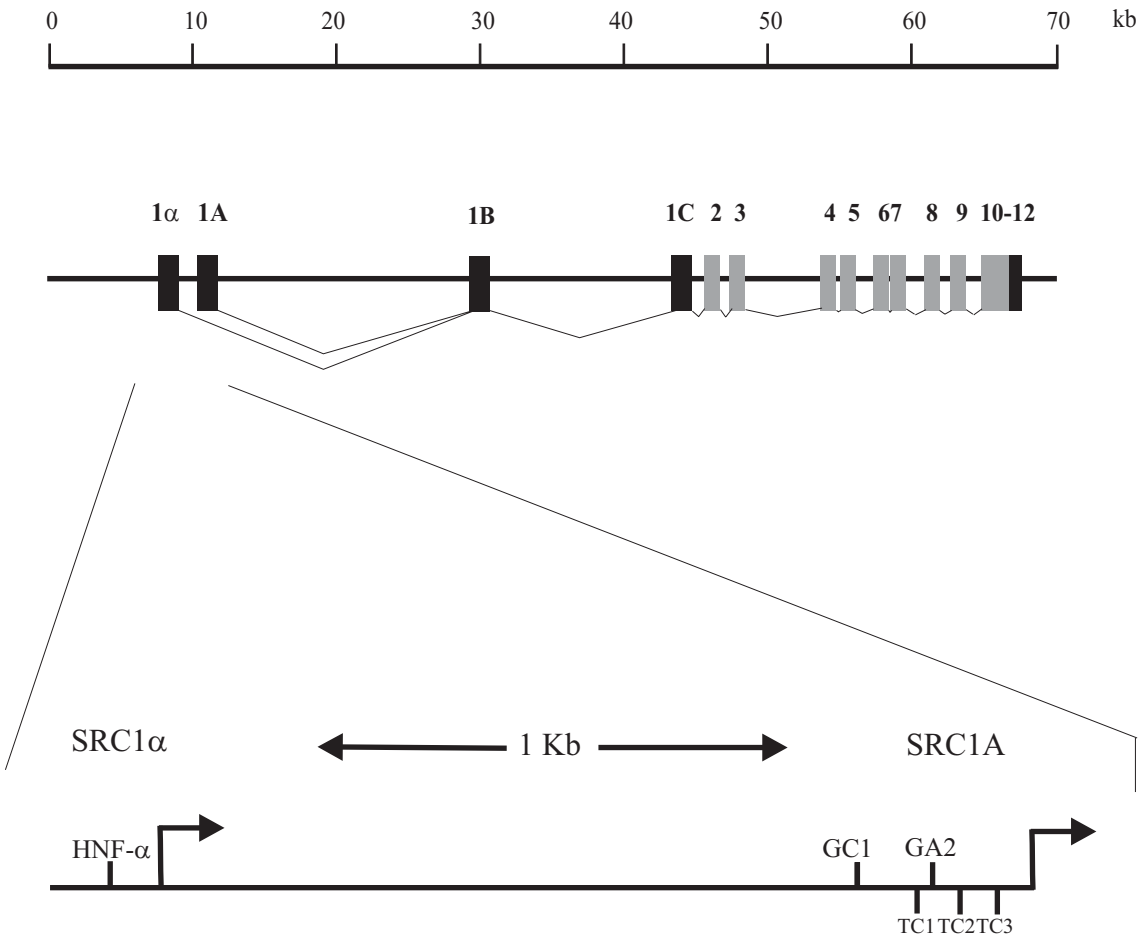


Figure 1.8. Architecture of SRC. The SRC exons are represented by the green or black rectangles (1α exon -exon 12). The black exons are untranslated regions and the grey exons are coding regions. The lines connecting exon 1α and exon 1B, as well as exon 1A and exon 1B, indicate alternative splicing based on promoter usage. The SRC1α and SRC1A exons are as indicated. The SRC1α promoter is regulated by HNF-1α. The SRC 1A promoter is regulated by the Sp family of factors, which bind to the GC1 and GA2 sites, as well as by hnRNP K which binds to TC1, TC2 and TC3.

complicate the issue, more recent transient transfection experiments have revealed that Sp3 is able to activate the SRC1A promoter, suggesting that further study into the role of Sp3 at the SRC1A promoter is required (Dehm, *et al*., 2004). Despite the confusion surrounding the role of Sp3 in SRC1A activation, it is clear that the interactions between the Sp family of transcription factors and SRC1A are essential to promoter activity as mutations abrogating binding of Sp factors to GC1 and GA2 reduced SRC1A activity by 70% (Ritchie, *et al.*, 2000). Similarly, individual deletions of the SRC1A TC1, TC2 and TC3 tracts also diminished SRC1A activity by 65%, 45% and 34% respectively indicating an essential role for hnRNP K binding in SRC1A activation (Ritchie, *et al.*, 2000).

hnRNP K is a complex protein with pleiotropic functions including: RNA splicing, transcriptional activation, chromatin remodeling, mRNA stability, and cellular signalling (Bomsztyk, *et al*., 2004). Specifically, hnRNP K has been reported to separate the double stranded DNA of a perfect polypyrimidine/ polypurine tract within the c-myc promoter to promote the formation of a single-stranded transcriptional bubble and contribute to transcriptional initiation (Michelotti, *et al*., 1996). At the SRC1A promoter, hnRNP K binds to double stranded TC1 and TC2 and to single-stranded TC1, 2 and 3 *in vitro*. Through mutation analysis, it has been observed that hnRNP K will only bind to the double-stranded tracts if the putative consensus sequence CTTCC is maintained. In addition, hnRNP K binds to the single-stranded tracts with greater affinity than to the double-stranded tracts and mutations that abolish single-stranded binding also abolish double-stranded binding *in vitro.* As a consequence of these findings, it has been suggested that hnRNP K participates in SRC1A transcriptional activation in a manner similar to that observed at the c-myc promoter. Specifically, binding of hnRNP K to the double-stranded TC1 and TC2 strands facilitates the separation of the double-stranded DNA as hnRNP K has a higher affinity for single-stranded TC1 and TC2. As a consequence of the strand separation, TC3 becomes single-stranded thus providing an additional hnRNP K binding target to mediate the movement of the transcription bubble towards the core promoter region and facilitate transcriptional initiation (Ritchie, *et al*., 2003). Furthermore, through the

characterization of the SRC1A promoter, it is clear that both the binding of Sp factors and hnRNP K are required for maximal SRC1A transcriptional activation.

1.5.2. SRC1α promoter

The SRC1α promoter was originally identified due to the observation that many SRC transcripts did not contain a 5'UTR originating from the SRC1A promoter. Subsequent experiments identified the SRC1α promoter as an alternative to the SRC1A promoter. Similar to the SRC1A promoter, SRC1α -mediated transcription is initiated through an Inr element. However, unlike SRC1A, the SRC1alpha promoter is regulated by the tissue specific, liver enriched transcription factor, Hepatocyte Nuclear Factor-1 alpha (HNF-1α) and, as a result, is characteristically active in stomach, kidney and pancreas (Bonham, *et al*., 2000). HNF-1α is a homeodomain transcription factor that binds to the GGTTAATNATTAAC(A/C) consensus sequence. The HNF-1α activation domain is located at the C-terminus of the protein and is comprised of three isolated regions that are absolutely essential for the role of HNF-1α in the activation of particular genes (Cereghini, 1996). In addition, HNF-1α can form homodimers or heterodimers with the related HNF-1β via a common dimerization domain, although, HNF-1α is a stronger transactivator than HNF-1β. In addition, the two family members differ structurally in activation domain components, thus suggesting a possible mechanism for differential activation by these two related factors (Cereghini, 1996; Hayashi, *et al*., 1999). At the SRC1α promoter, mutations that abolished HNF-1α binding resulted in a very dramatic decrease in SRC1α activity, further confirming a role for HNF-1α in SRC transcriptional activation (Bonham, *et al*., 2000). Significantly, while the SRC1α promoter contributes equally to mRNA transcribed from a variety of colon cancer cell lines and contributes a greater number of transcripts in HepG2 cells, the SRC1α promoter is consistently weaker than the SRC1A promoter in transient transfection analysis. This observation has led to the suggestion that the activity of the SRC1α promoter may be reliant on distal promoter elements such as enhancers (Dehm and Bonham, 2004b).

1.5.3. HDI -mediated transcriptional repression

Increased SRC expression has been observed in a variety of colon cancer and breast cell lines. Moreover, this increased expression was the result of transcriptional activation and correlated well with increased protein and kinase levels in these cell lines (Dehm, *et al*., 2001; Dehm and Bonham, 2004a). As described in a previous section, HDIs are potent chemotherapeutic drugs that are capable of deregulating transcription and influencing the activity of many genes. SRC is transcriptionally repressed by the HDIs, TSA and Sodium Butyrate. Moreover, although treatment with these drugs does not abrogate transcription factor binding to either SRC promoter, this repression does not require new protein synthesis and both the SRC1A and SRC1α promoters were repressed in a dose -dependent manner (Kostyniuk, *et al*., 2002). Attempts to identify an HDI responsive element common to both highly dissimilar promoters were fruitless. However, one common feature exists between the two SRC promoters. Both core promoters contain an Inr element and, as such, transcription is TAF1 -dependent. To further delve into the relationship between TAF1 dependency and HDI -mediated repression, a series of chimera constructs were produced. These constructs were chimeras of the SRC core promoter elements and the HDI inducible $p21^{WAF1}$ distal promoter elements and vice versa. Interestingly, all of the SRC/$p21^{WAF1}$ chimeric constructs were repressed by HDIs and -dependent on TAF1 (Dehm, *et al*., 2004). These results suggest a role for core promoter elements, and associated DNA specific transcription factors, in HDI -mediated repression.

2. SPECIFIC AIMS AND HYPOTHESIS

A consistent finding in a variety of cancers, including colon cancer, is an increase in c-Src protein levels followed by a corresponding increase in c-Src kinase activity. Members of the Bonham laboratory have demonstrated that the increased c-Src protein levels often correlate with increased SRC mRNA expression. As a result, the transcriptional regulation of SRC in human cancer cells is a source of significant interest to those attempting to elucidate the causative mechanisms of cancer. Interestingly, SRC expression is inhibited by the promising chemotherapeutic agents, known as histone deacetylase inhibitors. The mechanisms underlying this repression are unknown but could provide critical clues in determining how SRC expression is deregulated in cancer. Previous studies have suggested that these agents repress SRC by a mechanism involving both the core promoter and general promoter architecture. The basis for the hypothesis and specific aims of this thesis are drawn from the above observations.

HYPOTHESIS: Sp1 and Sp3 have contrary roles in SRC activation. Histone deacetylase inhibitor treatment results in decreased histone acetylation at SRC promoter regions. Histone deacetylases are required for SRC transcriptional activity.

SPECIFIC AIMS:

Specific Aim #1: To characterize the role of the SP family of transcription factors in SRC expression in human cancer cell lines.

Specific Aim #2: To determine the effect of histone deacetylase inhibitors on histone acetylation at the promoter regions of genes alternatively affected by histone deacetylase inhibitors. To characterize the impact of histone deacetylase inhibitors on transcriptional activation at the SRC promoter regions.

Specific Aim #3: To evaluate the role of histone deacetylases in SRC expression.

3. Materials and Methods

3.1. Reagents and Suppliers

The materials and reagents involved in this study are listed in Table 3.1 and are molecular biology or reagent grade. The commercially available kits used in this study are listed in Table 3.2. The equipment and software used in this study are listed in Table 3.3. The names and addresses of distributors are listed in Table 3.4.

Table 3.1. Materials and Reagent

Reagent	**Distributor Name**
Acetic acid (glacial)	BDH
Actinomycin D	Sigma-Aldrich Co.
Agarose	EMD Chemicals Inc.
Ampicillin	EMD Chemicals Inc.
β-Mercaptoethanol	EMD Chemicals Inc.
Bacto Agar	BD Biosciences
Boric acid	EMD Chemicals Inc.
Bromophenol blue	BDH
Calcium chloride dihydrate	BDH
Calf Intestinal Alkaline Phosphatase	New England Biolabs Ltd.
Chloroform	EMD Chemicals Inc.
Cycloheximide	Sigma-Aldrich Co.
Diethyl pyrocarbonate	Sigma-Aldrich Co.
Disodium hydrogen phosphate	EMD Chemicals Inc.
DMEM	Invitrogen – Gibco Cell Culture Systems
DMEM-F12	Invitrogen – Gibco Cell Culture Systems
DMSO	Sigma-Aldrich Co.
dNTPs	New England Biolabs Ltd.
EDTA	EMD Chemicals Inc.
Ethidium bromide	BDH
Fetal Bovine Serum	HyClone
Formaldehyde	EMD Chemicals Inc.

Formamide	BDH
GeneScreen Plus Hybridization Transfer Membrane	PerkinElmer Life Sciences, Inc.
Glycerol	EMD Chemicals Inc.
Glycine	EMD Chemicals Inc.
Guanidinium thiocyanate	BDH
Humulin	Eli Lilly Canada Inc.
Hydrochloric acid	EMD Chemicals Inc.
Isoamyl alcohol	BDH
Isopropanol	EMD Chemicals Inc.
Kanamycin	EMD Chemicals Inc.
Klenow Fragment	Fermentas Canada Inc.
Lambda DNA	GE Healthcare Bio-Sciences Inc.
Manganese chloride tetrahydrate	BDH
Magnesium chloride	BDH
Morpholinopropanesulfonic acid (MOPS)	Sigma-Aldrich Co.
N-Lauroyl sarcosine	Sigma-Aldrich Co.
O'GeneRuler 50 bp DNA Ladder	Fermentas Canada Inc.
O'GeneRuler 1 kb DNA Ladder	Fermentas Canada Inc.
o-nitrophenyl-β-D-galactopyranoside	Sigma-Aldrich Co.
pBluescript KS +	Stratagene
pCAT3-Basic	Promega
penicillin-streptomycin	Invitrogen – Gibco Cell Culture Systems
Pfu DNA Polymerase	Fermentas Canada Inc.
Phenol (water saturated)	EMD Chemicals Inc.
Potassium Acetate	BDH
Potassium Chloride	EMD Chemicals Inc.
Potassium dihydrogen phosphate	EMD Chemicals Inc.
Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories Ltd.
Rubidium Chloride	EMD Chemicals Inc.
Restriction endonucleases	New England Biolabs Ltd.
RPMI–1640	Invitrogen – Gibco Cell Culture Systems
Shrimp Alkaline Phosphatase	Fermentas Canada Inc.
Sodium acetate	BDH
Sodium butytrate	Sigma-Aldrich Co.
Sodium bicarbonate	EMD Chemicals Inc.
Sodium carbonate	EMD Chemicals Inc.
Sodium chloride	EMD Chemicals Inc.

Sodium citrate	BDH
Sodium dihydrogen phosphate	BDH
Sodium hydroxide	BDH
Sodium phosphate (monobasic)	EMD Chemicals Inc.
Sodium phosphate (dibasic)	EMD Chemicals Inc.
SuperFect Transfection Reagent	Qiagen Inc.
T4 DNA Polymerase	GE Healthcare Bio-Sciences Inc.
Taq DNA Polymerase	Qiagen Inc.
Trichostatin A	Sigma-Aldrich Co.
Tris	EMD Chemicals Inc.
Trypsin-EDTA 1X	Invitrogen – Gibco Cell Culture Systems
Xylene Cyanol FF	BDH

Table 3.2. Commercially available kits

Commercial Kit	**Distributor Name**
CAT ELISA Kit	Roche Diagnostics
EndoFree Plasmid Maxi Kit	Qiagen Inc.
FastPlasmid Mini Kit	Eppendorf AG
QIAfilter Plasmid Midi Kit	Qiagen Inc.
QIAprep Miniprep Kit	Qiagen Inc.
QIAquick Gel Extraction Kit	Qiagen Inc.
Quick Ligation Kit	New England Biolabs, Ltd.
Zero Blunt TOPO PCR Cloning Kit	Invitrogen

Table 3.3. Equipment and Software

Equipment and Software	**Distributor Name**
Biofuge 13 microcentrifuge	Thermo Electron Corporation - Heraeus
CO_2 Incubator 3326	Forma Scientific, Inc.
Coulter Counter ZM	Coulter Electronics, Ltd.
Gel Doc 2000	Bio-Rad Laboratories Ltd.
Gene Amp PCR System 2700	Applied Biosystems Canada
Horizontal Gel Electrophoresis System	Owl Separation Systems
Isotemp Incubator 230D	Fisher Scientific Company
JA-10 rotor	Beckman Coulter Canada, Inc.
J2-MI highspeed centrifuge	Beckman Coulter Canada, Inc.
MacVector 7.2.3	Accelrys Inc.
Microplate Reader Model 3550	Bio-Rad Laboratories Ltd.

Molecular Imager FX	Bio-Rad Laboratories Ltd.
ORBIT Incubator Shaker	Lab-Line
SmartSpec 3000 Spectrophotometer	Bio-Rad Laboratories Ltd.
Sorvall RT6000D	Du Pont Canada, Inc.
Quantity One Software, Version 4	Bio-Rad Laboratories Ltd.
UV Stratalinker 2400	Stratagene

Table 3.4. Names and Addresses of Distributors

Distributor Name	**Address**
Accelrys Inc.	Accelrys Inc., San Diego, CA, USA
Applied Biosystems Canada	Applied Biosystems Canada, Streetsville, ON, Canada
BarnsteadIThermolyne Corp. International	BarnsteadIThermolyne Corp., Dubuque, Iowa, USA
BD Biosciences	BD Biosciences, Mississauga, ON, Canada
BDH Inc.	BDH Inc., Toronto, ON, Canada
Beckman Coulter Canada, Inc.	Beckman Coulter Canada, Inc., Mississauga, ON, Canada
Bio-Rad Laboratories Ltd.	Bio-Rad Laboratories Ltd., Mississauga, ON, Canada
Coulter Electronics Ltd.	Coulter Electronics Ltd. Luton, Beds., England
Du Pont Canada, Inc.	Du Pont Canada, Inc., Mississauga, ON, Canada
Eli Lilly Canada Inc.	Eli Lilly Canada Inc., Scarborough, ON, Canada
EMD Chemicals Inc.	EMD Chemicals Inc., Gibbstown, NJ, USA
Eppendorf AG	Eppendorf AG, Hamberg, Germany
Fermentas Canada Inc.	Fermentas Canada Inc., Burlington, ON, Canada
Fisher Scientific Company	Fisher Scientific Company, Nepean, ON, Canada
Forma Scientific, Inc.	Forma Scientific, Inc., Marietta, OH, USA
GE Healthcare Bio-Sciences Inc.	GE Healthcare Bio-Sciences Inc., Baie d'Urfe, QC, Canada
HyClone	HyClone, Logan, UT, USA
Integrated DNA Technologies, Inc.	Integrated DNA Technologies, Inc., Coralville, IA, USA

Invitrogen Canada Inc.	Invitrogen Canada Inc., Burlington, ON, Canada
New England Biolabs, Ltd.	New England Biolabs, Ltd., Pickering, ON, Canada
Owl Separation Systems	Owl Separation Systems, Portsmouth, NH, USA
PerkinElmer Life Sciences, Inc.	PerkinElmer Life Sciences, Inc., Boston, MA, USA
Promega	Promega, Madison, WI, USA
Qiagen Inc.	Qiagen Inc., Mississauga, ON, Canada
Roche Diagnostics	Roche Diagnostics, Laval, QC, Canada
Sigma-Aldrich Co.	Sigma-Aldrich Co., Oakville, ON, Canada
Stratagene	Stratagene, La Jolla, CA, USA
Thermo Electron Corporation - Heraeus	Thermo Electron Corporation, Waltham, MA, USA

3.2. Tissue Culture and Cell lines

3.2.1. Cell lines and Standard Tissue Culture conditions

All cell lines utilized in this study were obtained from the American Type Culture Collection (ATCC). Tissue culture media required for these studies was obtained from Invitrogen. HT29 and SW480 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS, HyClone). HepG2 cells were grown in DMEM F12 supplemented with 10% FCS. The *Drosophila* SL2 cell line was grown in Schneider's media (GibcoBRL) supplemented with 10% FCS. All cells were maintained at 37°C and 5% CO_2, except for SL2 cells, which were maintained at room temperature and approximately 0.05% CO_2.

3.2.2. Histone Deacetylase Inhibitor Treatment

Exponentially growing cells were trypsinized and seeded such that they were between 50-70% confluent at time of treatment. Twenty-four hours post-seeding cells were treated with 1μM trichostatin A (TSA, Sigma) and harvested at various time points.

3.2.3. ChIP Tissue Culture

Exponentially growing HepG2 and HT29 cells were trypsinized and seeded such that they were between 50-70% confluent at time of treatment. Twenty-four hours post-seeding, cells were treated with 1μM TSA. At set time points, formaldehyde was added directly to growth media to a final concentration of 1% and cells were incubated at 37°C for 10 minutes. Cells were washed twice in ice cold Phosphate buffered saline (PBS, 0.15 M NaCl, 20 mM sodium phosphate, pH 7.4) containing freshly added protease inhibitor mix (PIM, Sigma), 1 μM pepstatin A, 1 M sodium vanadate and 1 M Sodium Fluoride. Cells were scraped off the plate, pelleted and flash frozen to be stored at -80°C until use. Generally, $5x10^6$ or $13\text{-}16x10^6$ cells were aliquoted and pelleted per tube to be used in an antibody -dependent manner.

3.3. Bacterial Strains

Escherichia coli (*E. coli*) strain TOP10 was used in the production of all plasmid vectors. Transformed *E. coli* cells were grown at 37°C in shaking incubator in Luria-Bertani (LB, 1% tryptone (w/v), 0.5% yeast extract (w/v), 1% sodium chloride (w/v), BD) broth supplemented with 100 μg/mL ampicillin or 34 μg/mL kanomycin (Fisher). LB-agar plates selective for transformed *E. coli* colonies were made with LB broth supplemented with 1.5% (w/v) agar and appropriate antibiotic. Plates were incubated overnight in a 37°C incubator.

3.4. General Molecular Biology Techniques

The majority of techniques used in this study are based on those outlined by Sambrook (Sambrook et al, 1989).

3.4.1. Molecular Cloning

3.4.1.1. Restriction Enzyme Digestion

All restriction enzymes used were obtained from New England Biolabs. Generally, digestions were performed with DNA at a concentration of 1 μg/μL and appropriate volumes of enzyme such that 1 μg DNA was incubated with 1 Unit of enzyme. The buffer used in these studies was One-Phor-All Buffer Plus (OPA, Amersham Biosciences) at appropriate concentrations as suggested by the

manufacturer. Reactions requiring complete digestion, were placed in 37°C incubator for a minimum of two hours, whereas, those requiring partial digestion were incubated at 37°C for a maximum of fifteen minutes. Partial digestion reactions were stopped with 6x loading dye (0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol FF, 30% glycerol (v/v) and 10 mM ethylenediamine tetracetic acid (EDTA)).

3.4.1.2. Polymerase Chain Reaction

Polymerase chain reactions (PCR) were performed with either *Taq* (Qiagen) or *Pfu* DNA polymerase (Stratagene), the buffers supplied with the enzymes, dNTPs (Amersham Biosciences) and specifically designed forward and reverse primers (Invitrogen). Concentrations of reaction components were determined by following manufacture instructions. Primers were designed using either MacVector or Vector NTI Advance 10 software inputed with target sequences; annealing temperatures were derived at time of primer design using the above software. Generally, sequences of interest were amplified by 30 cycles in the thermocycler (GeneAmp 2700, Applied Biosystems), with a melting temperature of 95°C and an extension temperature of either 68°C (*Pfu*) or 72°C (*Taq*). Extension times varied based on the size of the expected product and enzyme used; *Taq* driven extensions were determined using a 2 kb/min. rule and *Pfu* driven extensions determined using a 0.5 kb/min. rule.

3.4.1.3. Removal of 5' Terminal Phosphate

In order to dephosphorylate the 5' terminal ends of vector DNA prior to ligation, 1 Unit of calf intestinal alkaline phosphatase (Pharmacia Biotech) was added to restriction enzyme digests and incubated at room temperature for one hour.

3.4.1.4. Agarose Gel Electrophoresis

Agarose gels consisted of 0.75-2% agarose (w/v) (EMD) in TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH. 8.0) or TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH. 8.0). DNA fragments were loaded with 6X gel loading buffer into wells of agarose gel. Electrophoresis was performed for at least 45 minutes at 90 volts in either TAE or TBE buffer.

3.4.1.5. Purification of DNA Fragments

DNA separated by agarose gel electrophoresis for ligation reactions was cut out of the agarose gel and then isolated from the agarose using the MiniElute Gel Extraction Kit, the QIAquick Gel Extraction Kit or QIAEX II Gel Extraction Kit as per manufacture instructions (all kits available from Qiagen).

3.4.1.6. DNA Ligation

DNA ligations were performed using Quick T4 DNA ligase (New England Biolabs) and supplied buffer. Ligation reactions were generally performed using equal concentrations of purified dephosphorylated vector and purified insert (50 ng of vector and 50 ng of insert) in a final volume of 21 μL including supplied ligation buffer and enzyme. Reactions proceeded at room temperature for 13 minutes.

3.4.2. Site Directed Mutagenesis

All mutated constructs were obtained by using the QuickChange (Stratagene) site directed mutagenesis protocol with specifically designed primers that overlapped the site of mutation. Primers were designed using MacVector software.

3.4.3. DNA Sequencing

Automated DNA sequencing was performed either by Annette Kerviche at the Saskatchewan Cancer Agency using an ABI Prism 310 Genetic Analyser or by Inge Roewer at the PBI/NRC DNA Sequencing Lab using a 96-capillary AB 3730x/DNA sequence analyser.

3.4.4. Preparation and Transformation of Competent Cells

E. coli TOP10 cells were rendered competent for transformation prepared using a protocol described by Hanahan (Hananhan, 1983). Similarly, competent cells were transformed as described (Hananhan, 1983), plated onto LB plates supplemented with appropriate selective antibiotic and incubated overnight at 37°C.

3.4.5. Isolation of Plasmid DNA from Bacterial Cells

3.4.5.1. Small Scale Plasmid Purification

Isolated, transformed, bacterial colonies were picked from LB-agar plates, placed in 3 mL of LB-ampicillin/kanamycin culture media. Cultures were allowed to grow overnight at 37°C in the shaking incubator. The isolation of small quantities of plasmid DNA (<20 μg) from the cultures was performed by using either the

FastPlasmid Mini Kit (Eppendorf, VWR) or the QIAprep Spin Miniprep Kit (Qiagen) as per manufacturer's instructions.

3.4.5.2. Medium Scale Plasmid Purification

The isolation of moderate quantities of plasmid DNA (<100 μg) was performed by using the Plasmid Midi Kit (Qiagen). The isolation of plasmid DNA using this kit was performed as per manufacturer's instructions with the following exceptions: One mL of Buffer ER was added to the lysate after filtration of the lysate through the QIAfilter cartridge. Lysate was incubated on ice for 30 min. prior to filtration. All buffers used were of Endofree quality thus resulting in transfection quality plasmid DNA.

3.4.5.3. Large Scale Plasmid Purification

The isolation of large quantities of plasmid DNA (<500 μg) was performed using the Endofree Plasmid Maxi Kit (Qiagen) as per manufacturer's instructions. This kit provided plasmid DNA sufficiently pure to be utilized in mammalian transient transfection.

3.4.6. Isolation of RNA from Eukaryotic cells

RNA was isolated from dividing mammalian cultured cells as outlined by the guanidinium thiocyanate protocol reported by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Purified RNA pellets were resuspended in RNA storage buffer (0.1 mM EDTA, 0.1% (v/v) diethyl pyrocarbonate (DEPC)). Concentration and quality of isolated RNA was assessed by A_{260}/A_{280}. RNA with an A_{260}/A_{280} ratio of above 1.7 was considered acceptable for analysis.

3.4.7. Isolation of protein from Eukaryotic cells

Cultured mammalian cells were washed once in ice cold PBS and lysed directly in SDS loading buffer (65 mM Tris-HCL (pH. 7.0), 2% (w/v) SDS, 5 % β-mercaptoethanol, 10% (v/v) glycerol and 0.5% (w/v) bromophenol blue). Samples were processed by the movement of the samples through a 27.5 gauge syringe until no longer viscous. Samples were subsequently stored at -80°C.

3.4.8. Generation of radioactively labelled DNA Probes

Uniformly [P^{32}]-labelled radioactive probes suitable for Northern blot analysis were labelled through the random labelling of DNA fragments by the Ready-To-Go Labelled Beads (dCTP) kit (Amersham Biosciences) as per kit instructions.

3.4.9. Protein Concentration Determination

3.4.9.1. Bradford Method

The Bradford method was used to determine the protein concentration in cell lysates obtained from transient transfection studies. The assay required the addition of 10-15 μL cell lysate into 500 μL diluted Bradford reagent (Biorad). The reaction was incubated for ten minutes and then samples were read at A_{595}. Serial dilutions of bovine serum albumin (BSA) were assayed as protein standards.

3.4.9.2. Lowry Method

The Lowry method of protein quantification was used to assess the protein concentration of lysates obtained through cell lysis by SDS sample buffer. The protocol used was as outlined by manufacturer's instructions for the Total Protein Kit, Micro Lowry (Sigma, Peterson's modification).

3.5. Plasmid Construction

3.5.1. Expression Constructs

The CMV β-galactosidase (β-Gal) expression construct was a gift from Dr. W. Roesler (Biochemistry, University of Saskatchewan). The pBluescript II SK(+) was obtained from Fermentas. The *Drosophila* specific β-galactosidase (p97b) expression construct was a gift from Dr. L. Lania (University of Naples "Federico II," Naples, Italy). The SuPr-1 construct was a generous gift from Dr. L. Zon (Boston Children's Hospital, Boston, Massachusetts). HDAC (Histone deacetylase) 1 and 2 expression constructs were gifts from Dr. J. Davie (Manitoba Institute of Cell Biology, University of Manitoba). HDAC3 and 8 expression constructs were gifts from Dr. E. Seto (Moffit Cancer Centre, Tampa Bay, Florida). The HDAC5 expression vector was a gift from Dr. S. Khochobin (Institute Albert Bonniot, Grenoble, France). The HDAC6 and 10 expression vectors were kind gifts from Dr. Xiang-Jiao Yang (University of

McGill, Montreal, Quebec). The HDAC7 expression construct was a gift from Dr. E. Verdin (University of California, San Francisco, California).

3.5.2. CAT Reporter Constructs

The 0.38 SRC1A-CAT, 0.38 SRC1AGC1mut-CAT and 0.38 SRC1AGA2mut-CAT (CAT- chloramphenicol transferase) reporter constructs have been previously described (Bonham *et al*., 2000). The SRC1AΔGC1/GA2-GAL4-CAT has also been previously described (Dehm *et al*., 2004). The SRC1AGA2mutΔGC1-GAL4-CAT construct has been previously described (Ellis *et al*., 2006).

3.5.3. GAL4 Expression Constructs

All Sp1 and Sp3 expression vectors used in this study are depicted in Fig. 3.1. The pSG4-Sp3(GAL4Sp3ΔN(SG4)) and the pSG4-Sp3K539R(GAL4Sp3ΔNRmut(SG4)) were kind gifts from Dr. G. Gill (Harvard Medical School, Boston, Massachusetts). The pSG424(DNA binding domain (DBD) construct used in SG4 transfections) construct was obtained from Dr. R. O'Brien (Vanderbuilt University, Nashville, Tennessee). Plasmids pM(DBD construct used in all pM vector experiments), GAL4/Sp3(GAL4Sp3ΔN(pM)) and GAL4Sp1 were gifts from Dr. T. Sakai (Kyoto University, Kyoto, Japan). GAL4Sp3li was constructed by digesting both the pM vector and pPacSp3FL-NEW with *BamH*I. The subsequent fragment was inserted into the pM plasmid. GAL4Sp3liRmut and GAL4Sp3liQmut were produced by site-directed mutagenesis and the mutagenic primers specific for arginine (R) and glutamine (Q), respectively, as outlined above. The GAL4Sp3M1 construct was made by producing a PCR fragment with the following primers: sense (5'GGAATTCGCTATGGATAGTTCAGAC, *EcoR*I site inserted into primer) and anti-sense (5'ATGGATCCGCAGCTTCCACAGATGCCAG, *BamH*I site inserted into primer). The resulting PCR fragment and pM vector were digested with *EcoR*I and *BamH*I. The PCR fragment was then inserted into the vector. The GALSp3M2 construct was produced via PCR with the following primers: sense (5'GGATTCCAGACAATGACTGCAGGC, *EcoR*I site contained) and anti-sense (5'ATGGATCCGCAGCTTCCACAGATGCCAG, *BamH*I site contained). The product

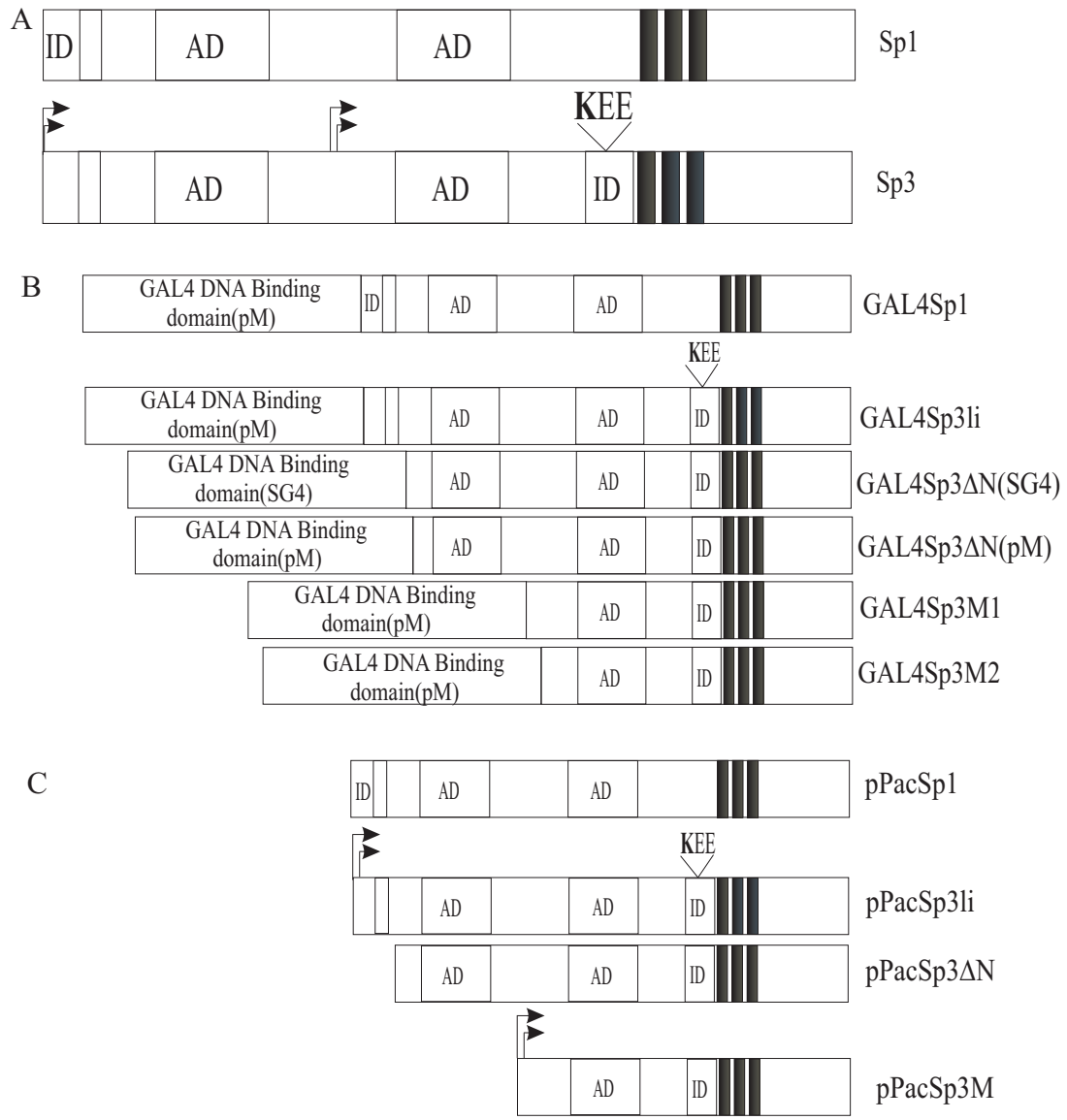


Figure 3.1. Sp1 and Sp3 constructs used in these studies. (A) The structure of Sp1 and Sp3. The black bands indicate the DNA binding domain, AD the activation domain, ID the inhibitory domain, N the amino terminal and C the carboxy terminal. The critical lysine residue present in all Sp3 isoforms is in bold with adjacent amino acids. The arrows indicate the translational start sites of Sp3. (B) The GAL4 expression constructs used to study Sp1 and Sp3 in human cells. All of the GAL4 Sp3 expression constructs were produced with a common pM vector backbone and fused to a GAL4 DNA binding domain unless otherwise noted. GAL4Sp3li construct produces one full length construct but is representative of both full length isoforms that are nearly equal in size and functionally identical. GAL4Sp3ΔN(SG4) expresses the Sp3ΔN isoform that is N-terminally truncated by ~100 amino acids and fused to the GAL4 DNA binding domain (DBD) of the SG424 construct. GAL4Sp3ΔN(pM) expresses the Sp3ΔN isoform (see above) but is fused to the GAL4 DBD of the pM construct. GAL4Sp3M1 and M2 are the shortest Sp3 isoforms derived from the GAL4 internal translation start sites and fused to GAL4 DBD of the pM construct. (C) The pPac expression constructs used for Sp1 and Sp3 analysis in *Drosophila* SL2 cells.

and plasmid pM were digested with *EcoR*I and *BamH*I and the resulting fragment inserted into vector pM. The GAL4Sp3M1R and Q mutants and GAL4Sp3M2R and Q mutants were produced by site-directed mutagenesis with the indicated primers as described above.

3.5.4. pPac Expression Constructs

The *Drosophila* specific pPacSp3(pPacSp3M), pPacUSp3(pPacSp3ΔN), pPacUSp3-K-mut(pPacSp3ΔNRmut), pPacSp3FL-NEW(pPacSp3li), and pPacSp3FL-NEW-K/R(pPacSp3MRmut) constructs were very generous gifts from Dr. G. Suske (Marburg University, Germany). pPacSp1 was a donation from Dr. R. Tijan (Howard Hughes Medical Institute, University of California). pPacSp3Rmut (arginine mutation) was created through site-directed mutagenesis using the following mutagenic primers: sense (5'GGATCAGAGAAGAAGAACCTGATCC) and
anti-sense (5' GGATCAGGTTCTTCTTCTCTGATCC).
pPacSp3MQmut and pPacSp3ΔNQmut (glutamine mutation) were made with the following mutagenic primers: sense (5'GGATCCAGGAAGAAGAACCTGATCC) and anti-sense (5' GGATCAGGTTCTTCTTCTCTGATCC).

3.6. Transient Transfection of Eukaryotic Cell Lines

3.6.1. Transfection with Reporter Constructs

All transfections were performed with plasmid DNA isolated from Qiagen's EndoFree Plasmid Maxi kit or the Plasmid Midi kit (Qiagen) modified to produce endofree plasmids and thus all transfected plasmids were endotoxin free.

3.6.1.1. Mammalian Cells

Generally**,** a typical co-transfection reaction consisted of 0.5-0.75 μg reporter (CAT) DNA, 0.5 μg CMV β-gal and 0-1 μg of plasmid DNA expressing the activator or repressor under study. The final concentration of DNA transfected into the cells was maintained at 2.0 μg by the addition of pBluescript. The DNA was mixed together in 85 μL serum free media; 10 μL of Superfect Transfection Reagent (Qiagen) was added and the reaction was incubated at room temperature for 20 minutes. During this incubation period, cells seeded at various confluencies the day before, were rinsed once in ice cold PBS. Subsequent to DNA complex incubation, 0.6 mL of serum-containing media was

added to the complexes, vortexed and added to cells. The transfections were allowed to proceed for 2.5 hours at which time 3 mL of serum-containing media was added to the cells. Cells were allowed to grow for an additional 48 hours prior to harvesting cell lysates. Specifically, in the GAL4 studies, 0.75 μg CAT-reporter DNA and 0.047-0.75 μg of GAL4DBD, GAL4Sp1 or GAL4Sp3 DNA was used. For the HDAC overexpression transfections, 0.5 μg reporter DNA and 0.25-1.0 μg HDAC DNA was used. Cells seeded 24 hours prior to transfection were seeded at the following confluencies: HepG2 cells at $2.5x10^5$/35 mm plate and SW480 cells at $5.0x10^5$/35 mm plate.

3.6.1.2. Insect Cells

All transfections into *Drosophila* SL2 cells were performed using Effectine reagent (Qiagen) at room temperature. Transfection mixtures were composed of 0.55 μg CAT reporter construct, 0.43 μg of p97b (*β*-galactosidase vector) and up to 85 ng of pPac expression construct. The final concentration of DNA transfected into the cell was maintained at 1 μg by the addition of pBluescript. In a typical transfection, 1 μg DNA was mixed with Buffer EC to a final volume of 100 μl, 8 μl Enhancer was added and mixtures were incubated at room temperature for 5 minutes. Following the incubation, 10 μl Effectine reagent was added to the mixture and incubated for a further 10 minutes. During this incubation period, cells seeded the previous day at $6x10^5$ per 35 mm plate were aspirated of media and 0.8 mL of new media was added per plate. Following the incubation, 0.6ml of media was added to the DNA/BufferEC/Enhancer/Reagent complexes, mixed and added to each well. The cells were grown for 48 hours at which time they were lysed.

3.6.2. Transfection with siRNA

All class I HDAC siRNA SMARTpools were obtained from Dharmacon RNA technologies (#M-003494-00-05, M-003495-00, M-003496-00-05, M-003500-00). HDAC 5, 6 and 10 siRNA was obtained from Ambion and HDAC 7 siRNA was obtained from Qiagen (#16708, 51320, 16708 and #S102777726). All siRNA specific for class II HDACs were specific for the HDAC in question and we were not given the target sequence. HT29 cells were seeded at $3x10^5$/ 35 mm plate and HepG2 cells were

seeded at $2.5x10^5$/ 35 mm plate 24 hours prior to transfection. In a typical siRNA transfection, 10 μL Lipofectamine 2000 (Invitrogen) was added to 0.5 mL OptiMEM (GibcoBRL) and incubated at room temperature for 15 minutes. After the incubation, 200 pmol of siRNA (final siRNA concentration of 100 nM) mixed with 0.5 mL OptiMEM was added to the reagent/media mixture and incubated for a further 15 minutes at room temperature. During this incubation, previously seeded cells were rinsed once with ice cold PBS and 1 mL of fresh complete media was added. Following the incubation, the siRNA/reagent complexes were added directly to the cells. Transfection was allowed to proceed for 6 hours at which time 2 mL of fresh complete media was added to the cells. Cells were harvested 48, 72 or 96 hours post-transfection. All transfection sets included mock (no siRNA) and scrambled siRNA (Dharmacon, Ambion) reaction.

3.7. Analysis of Reporter Gene Activity

Transiently transfected cells were rinsed once in ice-cold PBS 48 hours post-transfection and harvested in 500 μL 1 X lysis buffer available from Roche Applied Science.

3.7.1. β-Galactosidase Assay

A colorimetric assay described by Hall (Hall et al., 1983) was used to identify the uniformity of transfection efficiency among samples prior to performing CAT-ELISA studies. The reaction mixtures were placed at 37°C until a yellow colour developed. Colour development was halted by the addition of 120 μL of 1M Sodium Carbonate ($NaCO_3$, EMD Chemicals) stop solution. The sample absorbance was measured at a wavelength of 415 nm with a microplate reader.

3.7.2. Chloramphenicol Acetyltransferase Enzyme Linked Immunosorbant Assay (CAT ELIZA)

Levels of CAT expression in 200 μL of lysate were assayed using CAT-ELISA kit as per manufacturer's instructions (Roche Applied Sciences).

3.8. Western Blot Analysis

3.8.1. Western Procedure

Equal amounts of protein were resolved on a 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (PALL Life Sciences). Protein amounts resolved per well/gel varied depending on the experiment; 15 μg of protein isolated from siRNA transfected cells and 30 μg of protein isolated from transiently transfected cells.

3.8.2. Analysis of Western Blot with Chemiluminescence

Membranes were washed in TBST buffer (10 mM Tris, 15 mM NaCl, 0.5% (v/v) Tween-20) and blocked either at room temperature for 1 hour or at 4°C overnight in blocking buffer (5% (w/v) fat-free skim milk powder (Carnation) in TBST). Membranes were incubated for a minimum of one hour at room temperature in primary antibody diluted in blocking buffer. After incubation, membranes were washed thrice with TBST buffer and then incubated for a minimum of one hour at room temperature in secondary antibody (horsedradish peroxidase conjugate) diluted in blocking buffer. Subsequently, membranes were washed thrice in TBST buffer and incubated in chemiluminescent reagent (Pierce) at room temperature for one minute. Signal detection was achieved through exposure to Kodak X-Omat Blue XB-1 film. Primary antibodies used include: Sp3 (Santa Cruz Biotechnology, sc-644), HDAC 1 (05-614, Upstate), HDAC 2 (05-814, Upstate), Src (05-184, Upstate) and actin (Ab-1, Calbiochem). Host appropriate secondary antibodies were all obtained from Santa Cruz Biotechnology.

3.8.3. Analysis of Western Blot with LICOR Technology

Membranes were treated as recommended by LICOR Technologies (Western Blot Analysis Protocol). Host specific secondary antibodies were diluted 1:15,000 into Odyssey Blocking Buffer (LICOR Technologies) and were specifically obtained from LICOR Technologies. Primary antibodies used in these studies were identical to those used for Western Blotting with chemiluminesent detection.

3.9. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as described above and subjected to RT-PCR using Qiagen's One Step RT-PCR Kit as per manufacturer's instructions. RT-PCR primers

were designed using MacVector or Vector NTI Advance 10 software. The primers used in these studies were as follows:

Table 3.5. RT-PCR Primers

HDAC1	FOR: 5'-AAGGGGTGGCTGGGTCTTCAAGGA REV: 5'-ACAGAGGGCAGGCAGTGTTTCTTG
HDAC2	FOR: 5'-GCCACTGCCGAAGAAATG REV: 5'-ATGATGTAATCCTCCAGCCC
HDAC3	FOR: 5'-GAGTGGCTTGGGATGCTGTG REV: 5'-GGAGGAAGTCAGAGGCAATCTCAG
HDAC4	FOR: 5'-CCCCCACCAAGCCGAGGT REV: 5'-CGTCCGCGGATGCACTCG
HDAC5	FOR: 5'-ACAGGTGTGGTCTACGAC REV: 5'-TTTGCGACCTCGGATCCG
HDAC6	FOR: 5'-GGGCAGTCCCCTGAGGAGCGG REV: 5'-GCCTTCCGGGAAGCTGTC
HDAC7	FOR: 5'-GCAGCGCTCGGTGGAGCCCA REV: 5'-CGGGCGTGCTGCTACTAC
HDAC8	FOR: 5'-TTATGTGCTGGAAATCACGCC REV: 5'-CCTCCTGCCTACAAACTGGTG
HDAC9	FOR: 5'-TTAATTCAGTTGCAATTACCGCCAAATAC REV: 5'-TCAAACTCTTTGGCCACA
HDAC10	FOR: 5'-CCGGCTGCTCTGGGACGACC REV: 5'-CCGCGCGCAGTGAAAGGT
SRC	FOR: 5'-GGCTACATCCCCAGCAACTACG REV: 5'-CCCTTGAGAAAGTCCAGCAAACTC
RPL13A	FOR: 5'-CAAGGTGTTTGACGGCATCC REV: 5'-GCTTTCTCTTTCCTCTTCTCCTCC

The majority of the primers were designed to span intronic sequences to ensure that the amplified product of desired size was derived from mRNA transcript and not genomic DNA. Products were visualized using agarose gel electrophoresis.

3.10. Chromatin Immunoprecipitation (ChIP) Assays

Cell pellets isolated during ChIP tissue culture were subjected to ChIP analysis using Upstate's ChIP Assay Kit as per manufacturer's instructions. The following modifications were made to the kit protocol. Cell pellets were lysed in either 350 μL (for pellets containing $5X10^6$ cells) or 1 mL (for pellets containing $13\text{-}16X10^6$ cells) SDS Sample Buffer (Upstate) diluted with protease inhibitors (1 X PIM, 1 μM pepstatin A, 1M Sodium Vanadate and 1 M Sodium Fluoride). Genomic DNA was sheared for 35 seconds using a Branson Sonifier 450 sonicator (output control of 1.5, 60% duty cycle) to an average size of 300-800 bp. Cleared lysates were split into three fractions; input fraction, positive antibody fraction and negative antibody fraction. Subsequent to ChIP protocol, resultant genomic DNA was subjected to PCR analysis to identify factor enrichment at specific genomic loci. PCR products were analysed by agarose gel electrophoresis. The PCR primers used in these studies are described below:

Table 3.6. ChIP PCR Primers

SRC1alpha	FOR: 5'-GACAAGTCGATCAGCTTCC REV: 5'-GCAAGTAGGTAAGGGCCAG
SRC1A	FOR: 5'-AGGCGGATCTGGGGCGTAG REV: 5'-ATTCCGGGCCGGGAGAGAC
p21(#1)	FOR: 5'-GGGCGGGGCGGTTGTATATCAG REV:5'-GTCTGCCGCCGCTCTCTCACCT
p21(#2)	FOR: 5'-CGCACCAACGCAGGCGAGGG REV: 5'-ACGCTTGGCTCGGCTCTGGG
RPL13A	FOR: 5'-TCAGTCGCTTGAAGGGGTAATG REV: 5'-AAGGAGGAGGTTTTGTCGCAGG
Exon1A/1B	FOR: 5'-TCAGTGTGGCTTCAGTGAGGTG REV: 5'-CCTGGTGAGGTTTACCCAAAAAG

Exon1B/1C	FOR: 5'-CCATTGACTTGTCCCTCAGGAG REV: 5'-GGCAGGTTCCGATTATTCTTTGG
Exon4/5(#1)	FOR: 5'-TCCTACTCACCCTCCCATTC REV: 5'-CCTCCACCTGCCAGATAACTTCAC
Exon4/5(#2)	FOR: 5'-TGCTGAGTGCTTGAAGTTGCG REV: 5'-CCAGGTGAAAAAGGCAGAACC
3'UTR	FOR: 5'-TCAAACCCTGCCCTCCTTAGAC REV: 5'-CATCACCCACAAGCCGATTG

The antibodies used in these studies include: Sp3 (Santa Cruz Biotechnology, sc-644 and Upstate, 07-107), Sp1 (Santa Cruz Biotechnology, sc-420 and Upstate, 07-645), hnRNP K (a kind gift from Dr. Gideon Dreyfuss, Howard Hughes Medical Institute, Philadelphia, Pennsylvania), HDAC1 (05-614, Upstate), RNA Polymerase II (Upstate, 05-623 clone CTD4H8), RNA Polymerase II CTD Serine 2 Phospho-specific (Bethyl Laboratories Inc., BL2894), RNA Polymerase II CTD Serine 2 Phospho-specific (Bethyl Laboratories Inc., BL2896), Acetylated Histone H3 (Upstate, 06-599), Acetylated Histone H4 (Upstate, 06-866), Trimethylated Histone H3K9 (Upstate, 07-442), Acetylated Histone H3K9 (Upstate, 07-352), Acetylated Histone H3K14 (Upstate, 07-353), Acetylated Histone H4K5 (Upstate, 07-327), Acetylated Histone H4K8 (Upstate, 07-328), Acetylated Histone H4K12 (Upstate, 07-595) and Acetylated Histone H4K16 (Upstate, 07-329).

3.11. Real-Time PCR

All Real-Time PCR reactions were performed using the MiniOpticon System (Bio-Rad). Real-Time PCR was performed with the QuantiTect Multiplex No-ROX PCR Kit (Qiagen), which included buffers, enzymes and dNTPs. All suggested reaction conditions were followed including a set annealing temperature of 60°C and 50 cycles/ run. The forward and reverse primers (Invitrogen) and TaqMan Major Groove Binding (MGB) probes (Applied Biosystems) used were designed using either MacVector or Vector NTI Advance 10 software inputed with target sequences. The primers used in these reactions are listed below:

Table 3.7. Real-Time PCR Primers

SRC1A	FOR: 5'-AGGCGGATCTGGGGCGTAG REV: 5'-ATTCCGGGCCGGGAGAGAC
SRC1alpha	FOR: 5'-GACAAGTCGATCAGCTTCC REV: 5'-GCAAGTAGGTAAGGGCCAG
p21	FOR: 5'-GGGCGGGGCGGTTGTATATCAG REV:5'-GTCTGCCGCCGCTCTCTCACCT
RPL13A	FOR: 5'-TCAGTCGCTTGAAGGGGTAATG REV: 5'-AAGGAGGAGGTTTTGTCGCAGG
3'UTR	FOR: 5'-TCAAACCCTGCCCTCCTTAGAC REV: 5'-CATCACCCACAAGCCGATTG

The Taqman probes and probe specific fluorophore used in this study are included in the following table.

Table 3.8. Taqman Probes

SRC1A	TGCGGCGCCCTGGCGGAGTG	FAM
SRC1alpha	CAGGCTGGCTTCTGCTGTTGACTGGC	FAM
p21	CACGCGAGGTTCCGGGACCGGC	TET
RPL13A	TTCCACTCACAAACATGGCGGACAGAGCG	TET
3'UTR	TGAGGGACCCTTCGAGATCATCACTTCCTTGC	FAM

4. RESULTS AND DISCUSSION

4.1 REGULATION OF THE SRC1A PROMOTER BY Sp1 AND Sp3

4.1.1. Role of Sp1 and Sp3 in SRC Transcriptional Activation

In previous studies performed with the SRC1A promoter and the Sp family of transcription factors, Sp1 was described as a potent activator of the SRC1A promoter. Conversely, Sp3 was identified as being unable to activate SRC1A and could repress Sp1 -mediated SRC1A activation, presumably through competition for binding sites (Ritchie, *et al.*, 2000). In a subsequent study, Sp1 was still identified as an activator of SRC1A activity; however, Sp3 was also able to potently activate SRC1A. As opposed to the first Sp3 study, the second study utilized a GAL4Sp3 fusion construct that allowed the analysis of Sp3 in mammalian cells (Dehm, *et al*., 2004). Due to obvious disparities between the Sp3 results, the regulation of the SRC1A promoter by the Sp family of factors was revisited. To identify if Sp1 and Sp3 interact *in vivo* with the SRC1A promoter, chromatin immunopreciptation assays (ChIPs) were performed in the colon cancer cell line HT29 with antibodies specific for Sp1, Sp3 and hnRNP K (Fig. 4.1.). To ensure the specificity of the antibody used for the factor in question, PCR was also performed with primers specific to the SRC1α promoter (not shown). Sp1, Sp3 and hnRNP K were all found to bind to the SRC1A promoter, and not the SRC1α promoter, in HT29 cells. These results suggest that all three factors are important in SRC regulation.

Both Sp1 and Sp3 are abundant within the mammalian cell. Therefore to identify the effect of Sp1 and Sp3 on SRC1A activity, in a dose -dependent manner, the interference of endogenous levels of Sp1 and Sp3 needed to be eliminated. To that end, a SRC1A promoter construct was generated where the GC1 and GA2 Sp binding sites were replaced with GAL4 recognition sequences (Fig. 4.2B). The hepatocarcinoma cell line, HepG2, and the colon cancer cell line, SW480, were then co-transfected with Sp1 and Sp3 GAL4 fusion constructs (Fig. 3.1) and the SRC1A GAL4 binding site modified reporter construct. As expected, GAL4Sp1 was a very potent activator of SRC1A as

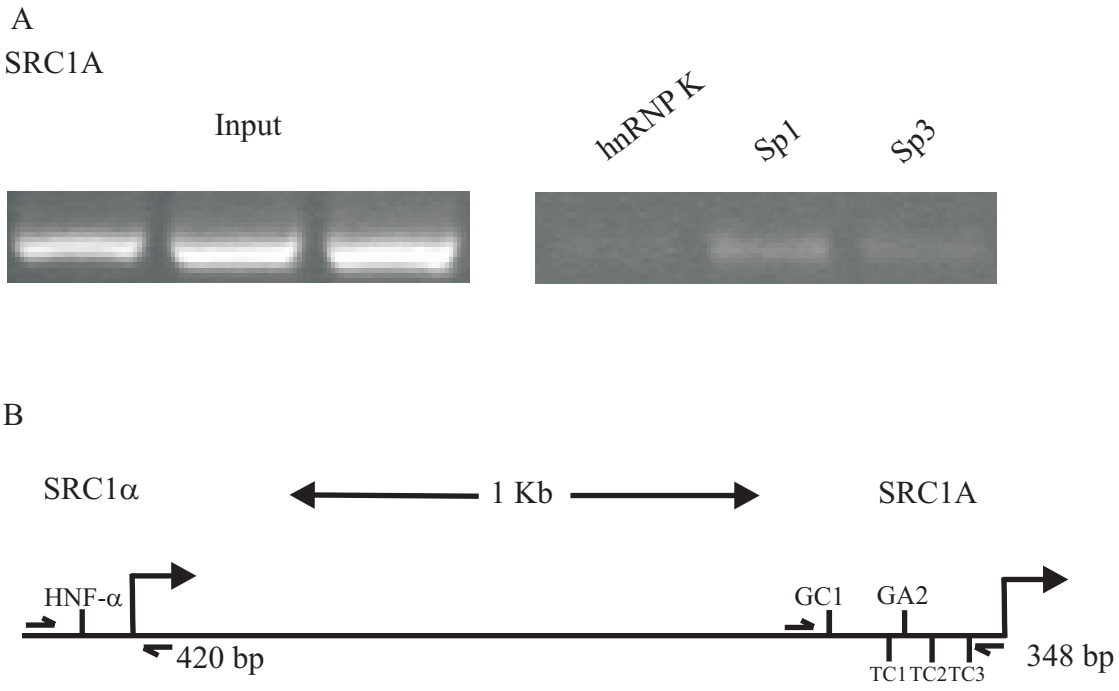
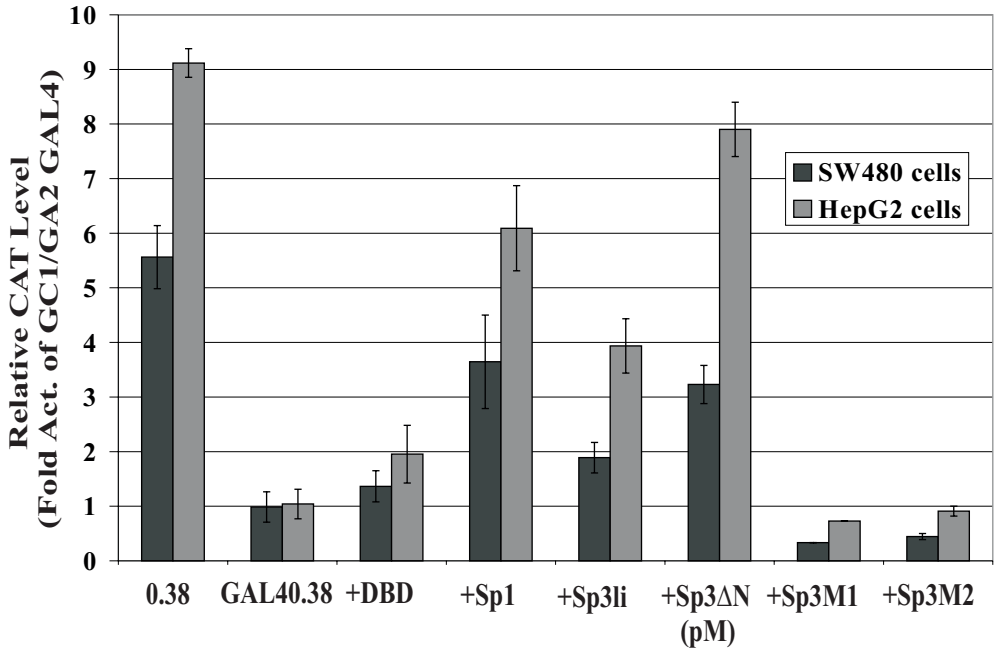


Figure 4.1. Sp1, Sp3 and hnRNP K bind the SRC1A promoter. (A) ChIP analysis was performed in HT29 cells with antibodies specific for Sp1, Sp3 and hnRNP K. Following immunoprecipitation and de-crosslinking, DNA was amplified with primers specific for the SRC1A promoter. This is representative of experiments performed at least three times (B) Graphical depiction of the primers used in the ChIP PCR analysis. A control PCR reaction was performed with primers specific to the SRC1α promoter (not shown).

A



B

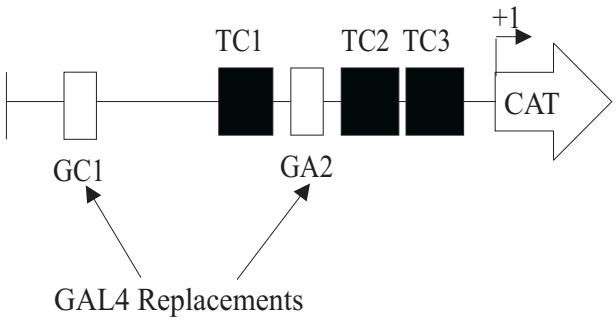


Figure 4.2. Role of Sp1 and Sp3 in SRC1A activation. (A) The SRC1AΔGC1/GA2-GAL4-CAT was co-transfected with Sp1 or Sp3 isoform GAL4 fusion construct into HepG2 or SW480 cells. DBD represents DNA binding domain activation alone. The error bars are representative of the standard deviation between two experiments each performed in duplicate. (B) The wildtype 0.38SRC1A promoter was mutated to replace the endogenous Sp binding sites with GAL4 binding sites (SRC1AΔGC1/GA2-GAL4-CAT).

compared to co-transfection with the DNA binding domain (DBD) alone (Fig. 4.2 A). However, GAL4Sp3li, which is equivalent to the newly discovered, physiologically relevant longest Sp3 isoforms, was a more modest activator of SRC1A (Sapetschnig, *et al.*, 2004). Conversely, the non-physiological GAL4Sp3ΔN(pM) construct was a far more potent activator of SRC1A than GAL4Sp3li, especially in HepG2 cells. This expression vector encodes a Sp3GAL4 fusion lacking approximately 100 amino acids from its N-terminal region. This non-physiological form of Sp3 has historically been considered as the full-length Sp3 and has been used in numerous studies (Sowa, *et al.*, 1999; Ross, *et al.*, 2002; Sapetschnig, *et al.*, 2002; Tang, *et al.*, 2004).

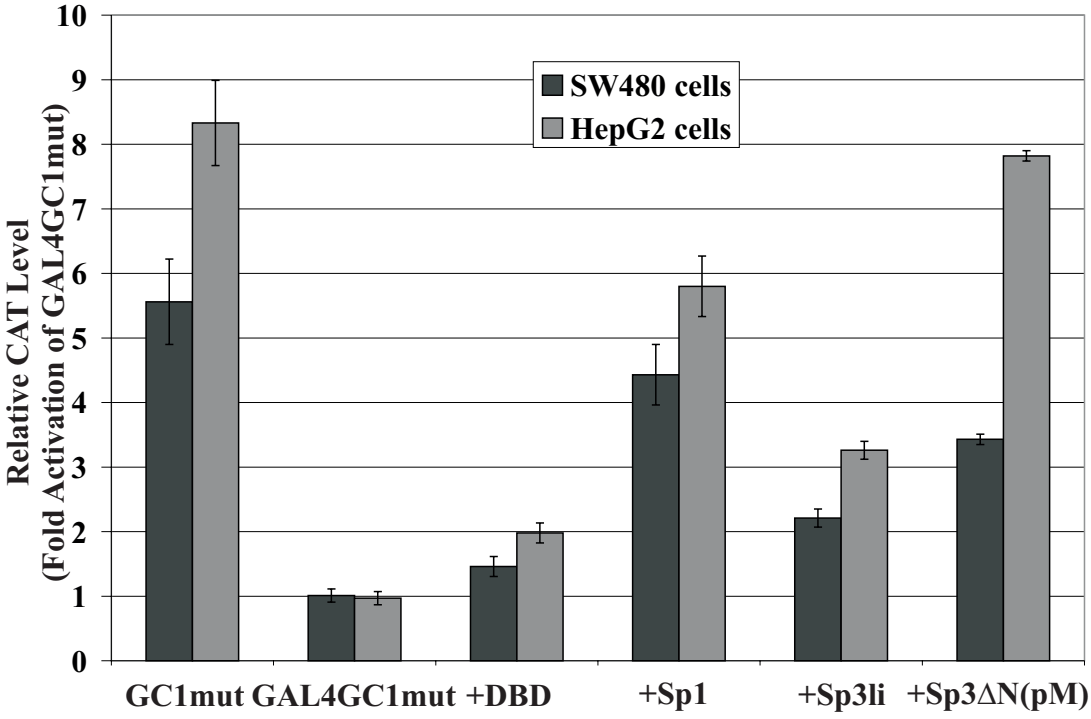
In contrast, the two shorter Sp3 isoforms, GAL4Sp3M1 and GAL4Sp3M2, were unable to activate the SRC1A promoter (Fig, 4.2. A). Though not shown, all of these constructs could impart a dose responsive effect, similar to what is shown in figure 4.2., on the SRC1A reporter construct in these cell lines. These results suggest that both Sp1 and Sp3ΔN are potent activators of SRC, whereas, Sp3li is a modest activator of SRC. In contrast, both short Sp3 isoforms are unable to activate SRC in mammalian cells.

4.1.2. Role of Binding Site and Cell Line in Sp Factor SRC1A Activation

The SRC1A promoter has two Sp binding sites, GC1 and GA2 (Ritchie, *et al.,* 2000). In order to determine if the effect of Sp1 and Sp3 on SRC1A activity is dependent on which binding site the factor is bound to, mutated reporter constructs were generated. To accomplish this, a SRC1A reporter construct was designed where the GC1 site was mutated and the GA2 site was replaced with a GAL4 recognition site (Fig. 4.3 B). In both HepG2 and SW480 cells, Sp1 was a potent activator of SRC1A via the GA2 site (Fig. 4.3 A). Both Sp3li and Sp3ΔN could activate SRC1A in both HepG2 and SW480 cells at the GA2 site. However, again, Sp3ΔN was a far more potent activator than full-length Sp3li.

To identify how Sp1 and Sp3 interacted with the GC1 site of SRC1A, a SRC1A reporter construct was modified such that the GA2 site was mutated and the GC1 site was replaced with the GAL4 recognition sequence (Fig. 4.4 B). In SW480 cells, Sp1, Sp3li and Sp3ΔN activated SRC via this site in a fashion similar to what was observed when the GA2 site was isolated (Fig. 4.4 A). However, the fold activation was

A



B

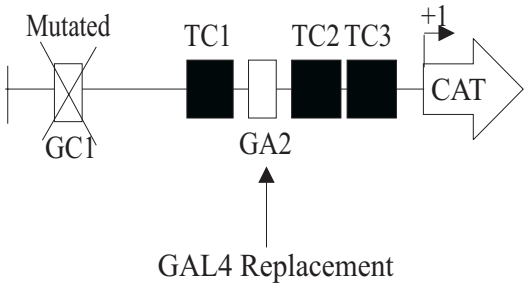


Figure 4.3. Role of binding site and cell line in Sp factor activation. (A) The SRC1AGC1mutΔGA2-GAL4-CAT was transactivated by Sp1 and long Sp3 isoform GAL4 fusion constructs in HepG2 and SW480 cells. (B)The 0.38GC1mutSRC1A-CAT promoter was mutated to replace the GA2 binding site with a GAL4 bindng site (SRC1AGC1mutΔGA2-GAL4-CAT). The error bars are representative of the standard deviation between two experiments each performed in duplicate.

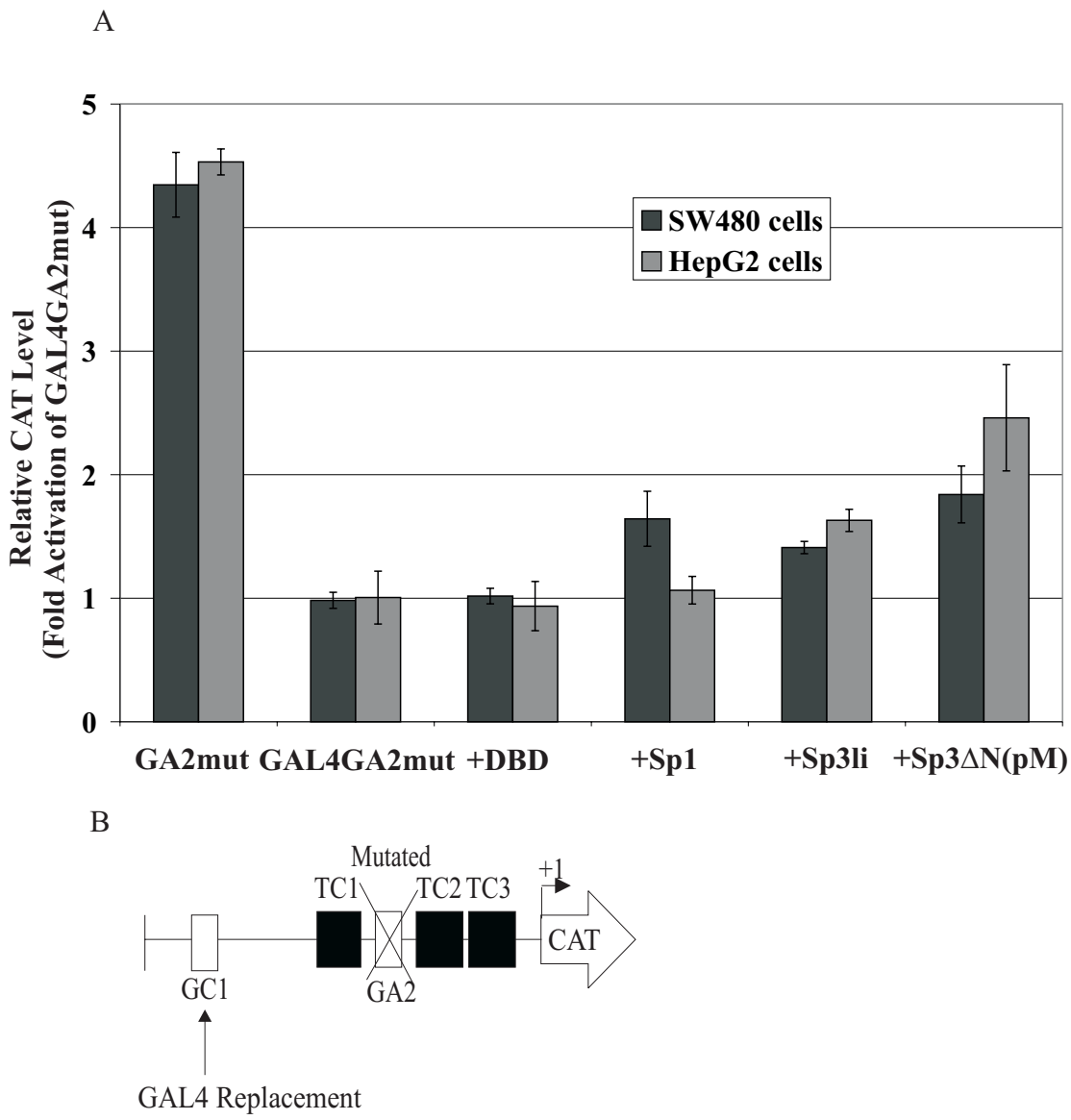


Figure 4.4. Role of binding site and cell line in Sp factor activation. (A) The SRC1AGA2mutΔGC1-GAL4-CAT was transactivated by Sp1 and long Sp3 isoform GAL4 fusion constructs in HepG2 and SW480 cells. (B) The 0.38GA2mutSRC1A-CAT promoter was mutated to replace the GC1 binding site with a GAL4 binding site (SRC1AGA2mutΔGC1-GAL4-CAT). The error bars are representative of the standard deviation between two experiments each performed in duplicate.

significantly reduced when activation via the GA2 site versus the GC1 site was compared. Similarly, in HepG2 cells, both Sp3li and Sp3ΔN activated SRC at the GC1 site. Again, Sp3ΔN was a more effective activator than Sp3li. Interestingly, Sp1 was unable to activate SRC via the GC1 site in HepG2 cells. Taken together, these results suggest that promoter activation by Sp1 or Sp3 is -dependent on both the architecture of the promoter and the cell line used. Furthermore, Sp1 is a potent activator of the SRC1A promoter via the GA2 and GC1 sites in SW480 cells but only via the GA2 site in HepG2 cells.

4.1.3. Sp1 and Sp3 -mediated SRC1A activation in *Drosophila* SL2 cells

To support the results observed with the GAL4 system in human cells, a series of transfections were performed in *Drosophila* SL2 cells. These cells are ideal for Sp factor studies as these cells do not express Sp1 and Sp3. Therefore, the endogenous abundance of Sp1 and Sp3 does not interfere with transient co-transfection studies and the effect of these factors on the native SRC1A promoter can be studied. Sp1 and Sp3ΔN were strong activators of SRC1A through binding at either the GC1 or GA2 Sp sites (Fig. 4.5). Sp3li was a comparatively modest activator of SRC1A activity, especially through the GC1 site. These results are consistent with the observations obtained from mammalian cells with the GAL4 system (Fig. 4.2 A, 4.3 A, 4.4 A). In contrast, Sp3M, which expresses the two shortest Sp3 isoforms, did not activate SRC1A at either binding site in SL2 cells, which is also consistent with observations using the mammalian GAL4 system (Fig.4.2 A).

4.1.4. Impact of Sp3 Modifications on SRC1A Activity in Mammalian Cells

A critical lysine residue (K551) within the inhibitory domain of Sp3 has been reported to be post-translationally modified by acetylation or SUMOylation. These studies have suggested that modification of this residue alters Sp3 -mediated activity, either through the recruitment of co-activators or co-repressors or the alteration of cellular localization (Braun, *et al*., 2001; Ross, *et al*., 2002; Verger, *et al*., 2004). In the interest of identifying how these modifications affect Sp3 -mediated SRC1A activation,

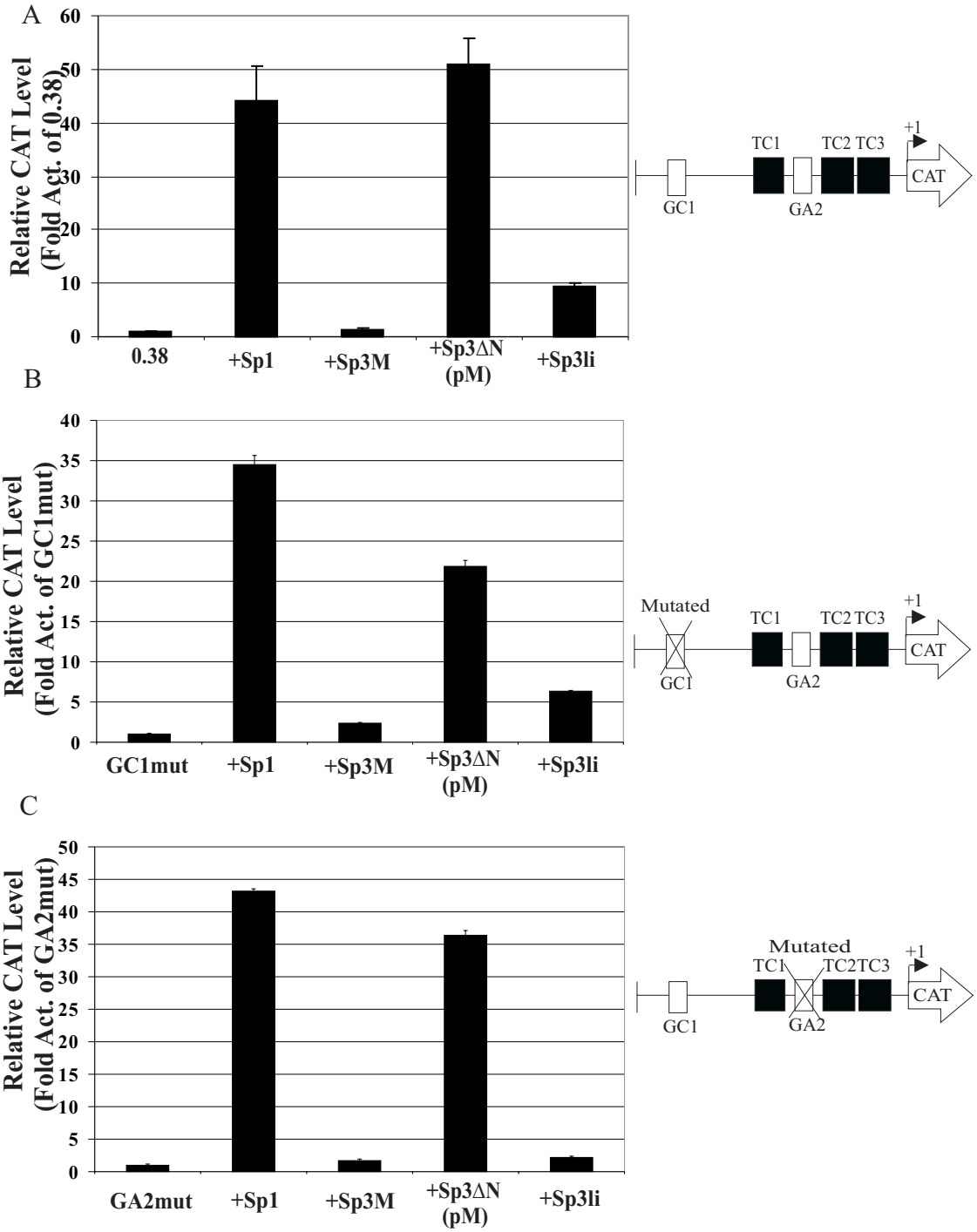


Figure 4.5. Sp1 and Sp3 SRC1A activation in SL2 cells. (A) The effect of the Sp1 and Sp3 isoforms was assayed using a wildtype 0.38*SRC*1A-CAT reporter construct in *Drosophila* SL2 cells. (B) The effect of the Sp1 and Sp3 isoforms was assayed using a 0.38GC1mutSRC1A-CAT reporter construct in *Drosophila* SL2 cells. (C)The effect of the Sp1 and Sp3 isoforms was assayed using a 0.38GA2mutSRC1A-CAT reporter construct in *Drosophila* SL2 cells. The Sp3M construct expresses both forms of the shortest Sp3 isoforms. The error bars are representative of the standard deviation between two experiments each performed in duplicate.

the GAL4 system was again utilized in mammalian cells (Fig. 4.6 A). SW480 and HepG2 cells were co-transfected with the GAL4 reporter construct and K551 modified versions of GAL4Sp3li. The first modification tested was a lysine to arginine substitution at residue 551 (K551R). This modification has been reported to prevent both acetylation and SUMOylation at this pertinent residue (Ross, *et al*., 2002) Interestingly, Sp3liRmut SRC1A activation was no different to wildtype Sp3li SRC1A activation in mammalian cells (Fig. 4.6. B). However, upon Sp3ΔNRmut transfection into SW480 cells, a two-fold increase in SRC1A activation was observed as compared to the wild type Sp3ΔN control (Fig. 4.6 C).

The effect of histone deacetylase inhibitors (HDI) treatment on Sp3ΔN -mediated SRC1A activation was also studied. HDIs repress SRC expression through an unknown mechanism (Kostyniuk, *et al*., 2002). Furthermore, the identification of Sp3 as an activator of SRC1A combined with the observation that upon Sp3 acetylation Sp3 is no longer an effective transcriptional activator suggests a potential mechanism for HDI -mediated SRC repression through Sp3 acetylation (Braun, *et al.*, 2001). If the acetylation of Sp3 did cause HDI -mediated SRC1A repression, it would be expected that SRC1A activation by the mutated form of Sp3ΔN would not change upon TSA treatment. Upon treatment with TSA, however, Sp3ΔNRmut activated SRC1A was not immune to HDI treatment and reporter activity decreased (Fig. 4.6 C). These results indicate that HDI -mediated SRC1A repression is not achieved through Sp3 acetylation.

The key lysine residue within the shortest GAL4Sp3 constructs were also modified to encode an arginine residue (GAL4Sp3M1Rmut and GAL4Sp3M2Rmut) and co-transfected into mammalian cells with the SRC1AGAL4 reporter construct. Interestingly, the results from the use of the shorter constructs were dramatically different from the results obtained with the longer constructs (Fig. 4.7. B, C). Both of the short Sp3 isoforms (Sp3M1, Sp3M2) were converted into very potent activators of SRC1A when both acetylation and SUMOylation were prevented (Sp3M1Rmut, Sp3M2Rmut). These results suggest that the modification of the shortest Sp3 isoforms by either SUMOylation or acetylation dampens the activating potential of these isoforms.

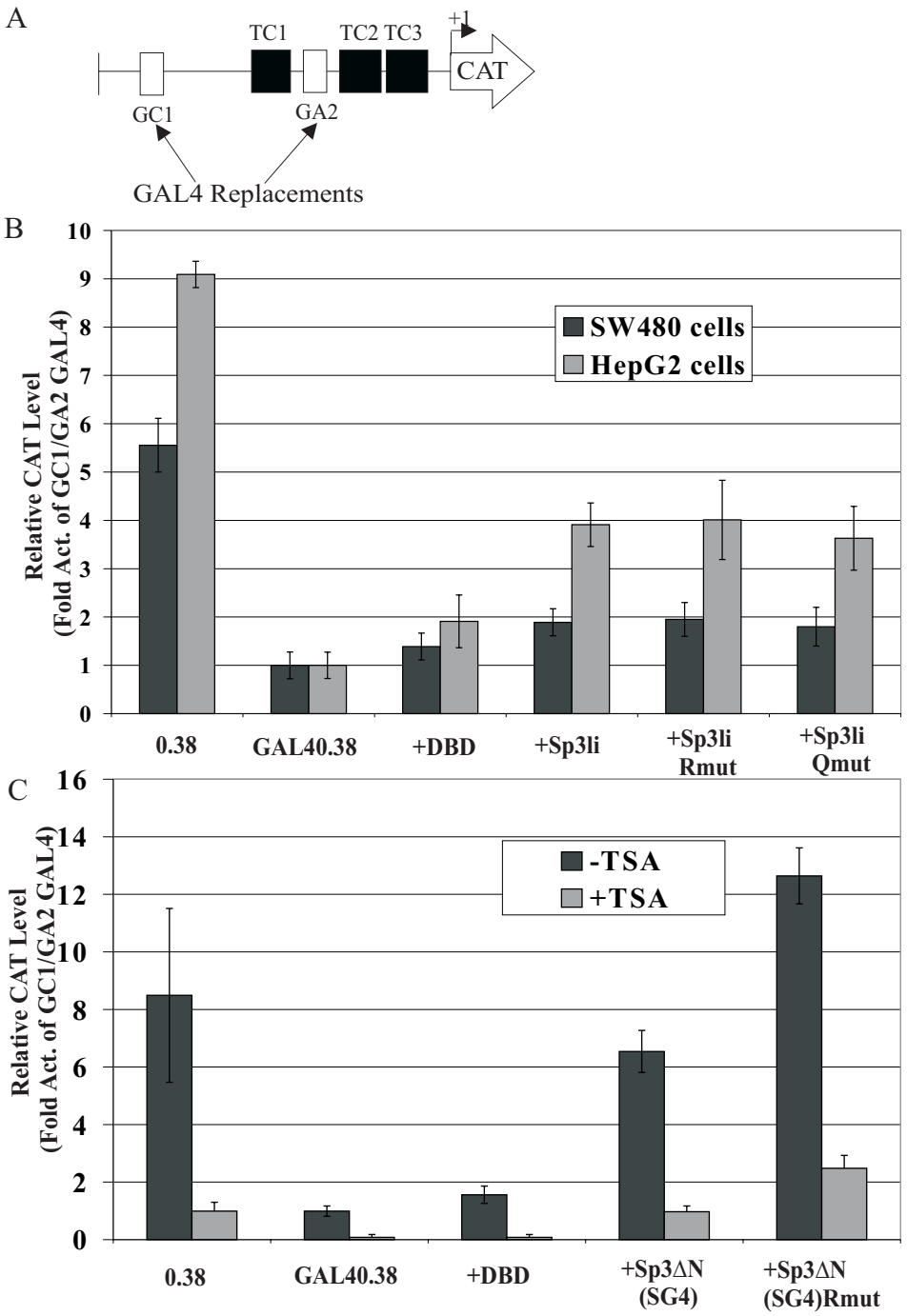


Figure 4.6. Role of SUMOylation and/or acetylation on Sp3 mediated SRC1A activation in mammalian cells. (A) The wildtype 0.38SRC1A promoter was mutated to replace the endogenous Sp binding sites with GAL4 binding sites (SRC1AΔGC1/GA2-GAL4-CAT). (B) The differences in GAL4Sp3li mediated SRC1A transactivation when comparing wild-type GAL4Sp3li, GAL4Sp3liRmut and GAL4Sp3liQmut in HepG2 and SW480 cells. (C) The differences in GAL4-Sp3ΔN(SG4) and GAL4Sp3ΔNRmut mediated SRC1A transactivation in SW480 cells with treament of 1μM TSA.The error bars are representative of the standard deviation between two experiments each performed in duplicate.

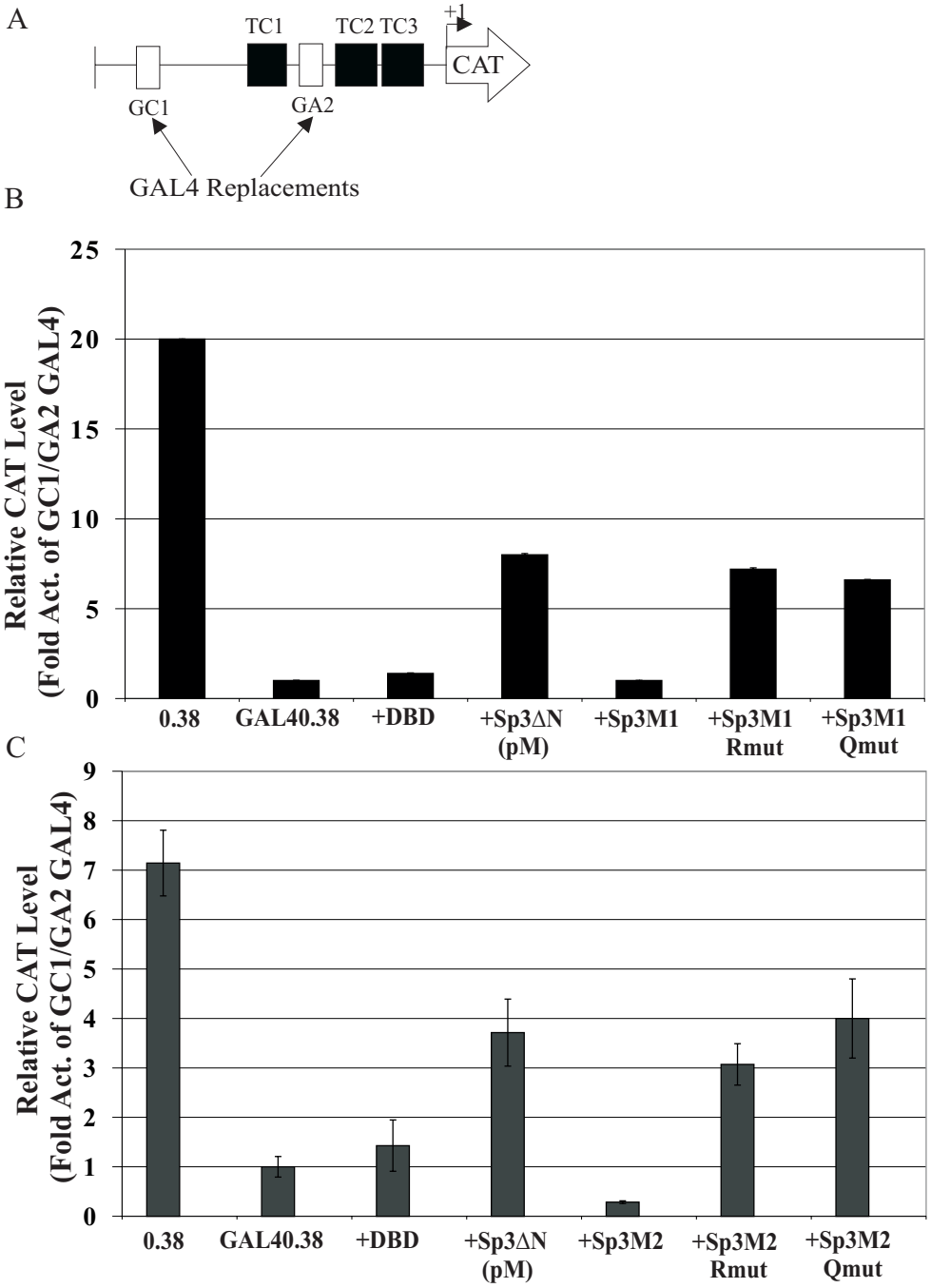


Figure 4.7. Role of SUMOylation and/or acetylation on Sp3 mediated SRC1A activation in mammalian cells. (A) The wildtype 0.38SRC1A promoter was mutated to replace the endogenous Sp binding sites with GAL4 binding sites (SRC1AΔGC1/GA2-GAL4-CAT). (B) The differences in GAL4Sp3M1, GAL4Sp3M1Rmut and GAL4Sp3M1Qmut mediated SRC1A transactivation in HepG2 cells. (C) The differences in GAL4Sp3M2, GAL4Sp3M2Rmut and GAL4Sp3M2Qmut mediated SRC1A transactivation in HepG2 cells.The error bars are representative of the standard deviation between two experiments each performed in duplicate.

To identify which modification was dampening the activational activity of Sp3M1/M2, the critical lysine residue was mutated to a glutamine residue (K551Q). A modification of this kind serves to mimic an acetylated lysine residue thus aiding in identifying which modification is affecting activational ability (De Nadal, *et al*., 2004). The longer Sp3 isoform was also similarly modified to act as a negative control. In agreement with previous Sp3liRmut results, Sp3liQmut demonstrated no difference in SRC1A activation as compared to the wildtype Sp3li in mammalian cells (Fig. 4.6. B). Significantly, the Sp3M1 and Sp3M2 constructs bearing the Q mutation were equally potent activators as the constructs bearing the R mutation thus suggesting that acetylation of Sp3M1/M2 has little impact on the potential for these factors to activate SRC1A.

Taken together, these results suggest that the modification of the longer forms of Sp3 have little effect on the ability of Sp3 to activate SRC1A in mammalian cells. Conversely, the modification of the shorter Sp3 isoforms by SUMOylation has a negative impact on the ability of these shorter isoforms to activate SRC1A in mammalian cells. Furthermore, Sp3 acetylation does not account for HDI -mediated SRC repression.

4.1.5. Role of SUMOylation on Sp3 -mediated SRC1A Activation in SL2 Cells

To complement the GAL4Sp3 SUMOylation and acetylation studies performed in mammalian cells, the effect of SUMOylation and acetylation on Sp3 activation potential was also studied using the *Drosophila* SL2 system. In SL2 cells, both the Sp3liRmut and wild-type Sp3li constructs modestly activated the SRC1A promoter (Fig. 4.8. B). Similarly, Sp3ΔN, Sp3ΔNRmut and Sp3ΔNQmut also activated SRC1A equally in SL2 cells. In contrast, Sp3MRmut and Sp3MQmut were significantly stronger SRC1A activators as compared to the wild-type Sp3M in SL2 cells. These results are consistent with that which was observed in mammalian cells, whereby modification of the longer forms of Sp3 had no affect on activation potential, however, modification of the shorter forms substantially increased the ability of Sp3M to activate the SRC1A promoter. To ensure equal expression of the Sp3 constructs, Western blots were performed with cell lysates obtained from SL2 cells transfected with the Sp3

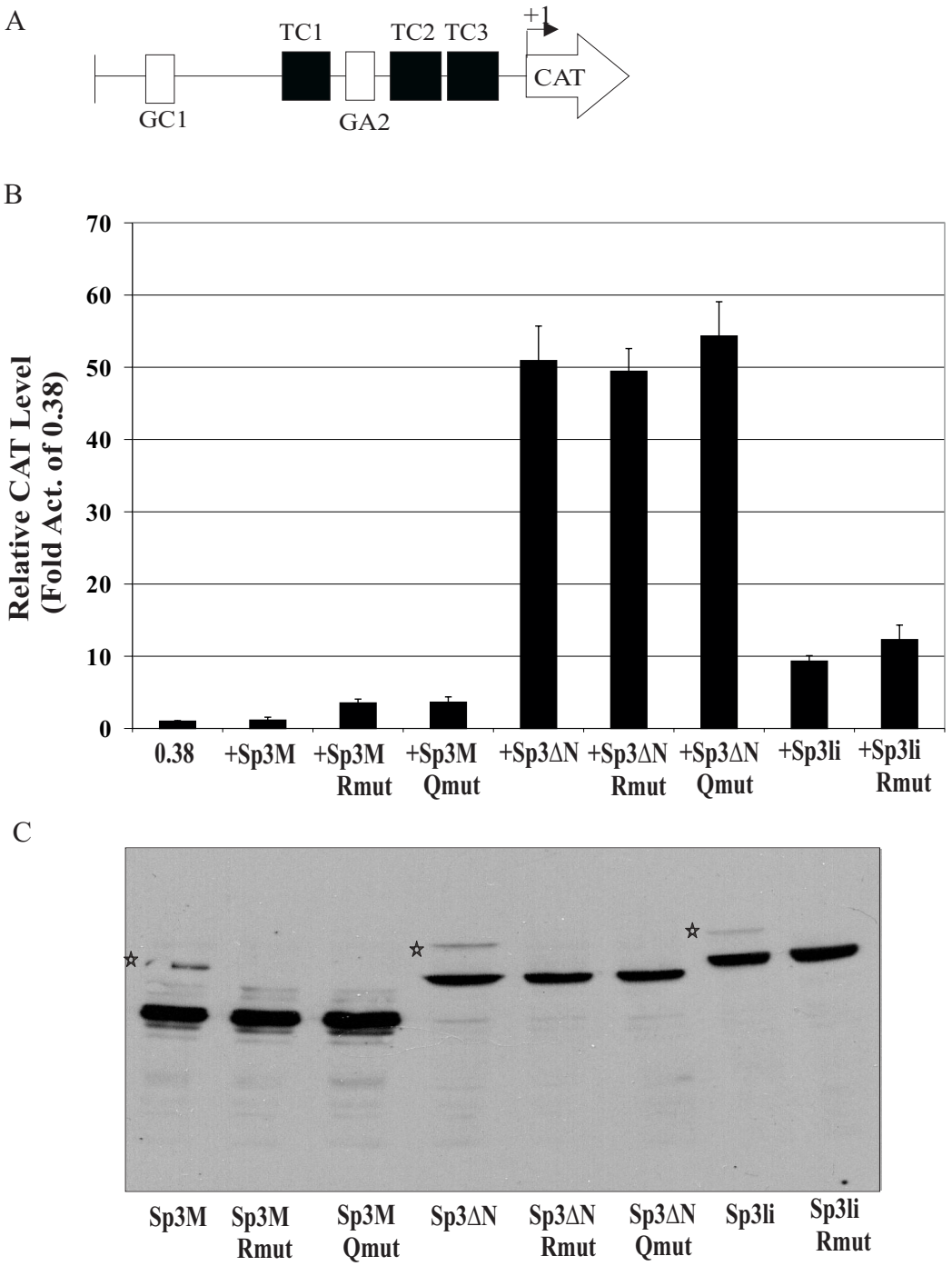


Figure 4.8. Role of modification on Sp3 mediated SRC1A activation in SL2 cells. (A) The wildtype 0.38SRC1A-CAT reporter construct used in the experiments in B and C. (B) Differences in Sp3 mediated SRC1A activation when comparing all wild-type Sp3 constructs and modified counterparts. The error bars are representative of the standard deviation between two experiments each performed in duplicate. (C) Western Blot analysis of all Sp3 isoforms and corresponding mutants from co-transfected SL2 cell lysates using an antibody specific for Sp3. Star represents wild-type Sp3 population that has been modified by SUMOylation.

constructs and an antibody specific to all isoforms of Sp3 (Fig. 4.8. C). These control experiments also served to confirm that the wild-type Sp3 constructs were indeed being modified by SUMO-1. The SUMOylated form of Sp3 can be seen migrating ~20 KDa above the non-SUMOylated forms of Sp3. The absence of the slower migrating Sp3 band in the lanes containing only mutated forms of Sp3 confirmed that the mutated forms of Sp3 were not SUMOylated.

These results are consistent with the results obtained from mammalian cells using the GAL4 expression constructs. Taken together, these results suggest that neither the SUMOylation or acetylation status of the long Sp3 isoforms affects the activation of SRC1A by Sp3li. In contrast, the SUMOylation, but not acetylation, of the shortest Sp3 isoforms cripples the activation potential of these isoforms. Furthermore, preventing the SUMO-1 modification of the shortest isoforms converts these factors into powerful activators of the SRC1A promoter.

4.1.6. SUMOylation of the Short Sp3 Isoforms Prevents SRC1A Activation

To further investigate if the SUMOylation of the shorter Sp3 isoforms was negatively impacting the ability of these isoforms to activate SRC1A, a construct expressing SUMO protease 1 (SuPr-1) was obtained. SuPr-1 specifically cleaves the SUMO-1 moiety from a target lysine residue (Splenger, *et al*., 2005; Ross, *et al.,* 2002). Co-transfection experiments in HepG2 cells were again performed, using the SRC1A GAL4 construct, GAL4Sp3M1, GAL4Sp3M2, and increasing levels of the SUMO-1 protease expression construct. The expression of SuPr-1 led to an increase in both Sp3M1 and Sp3M2 -mediated SRC1A activation (Fig. 4.9. B, C).

These studies further support that the role of the shorter Sp3 isoforms in the activation of SRC1A is directly -dependent on the SUMOylation status of the inhibitory domain. While SUMOylated Sp3M1 and Sp3M2 are ineffective activators of SRC1A, non-SUMOylated Sp3M1 and Sp3M2 are powerful SRC1A activators.

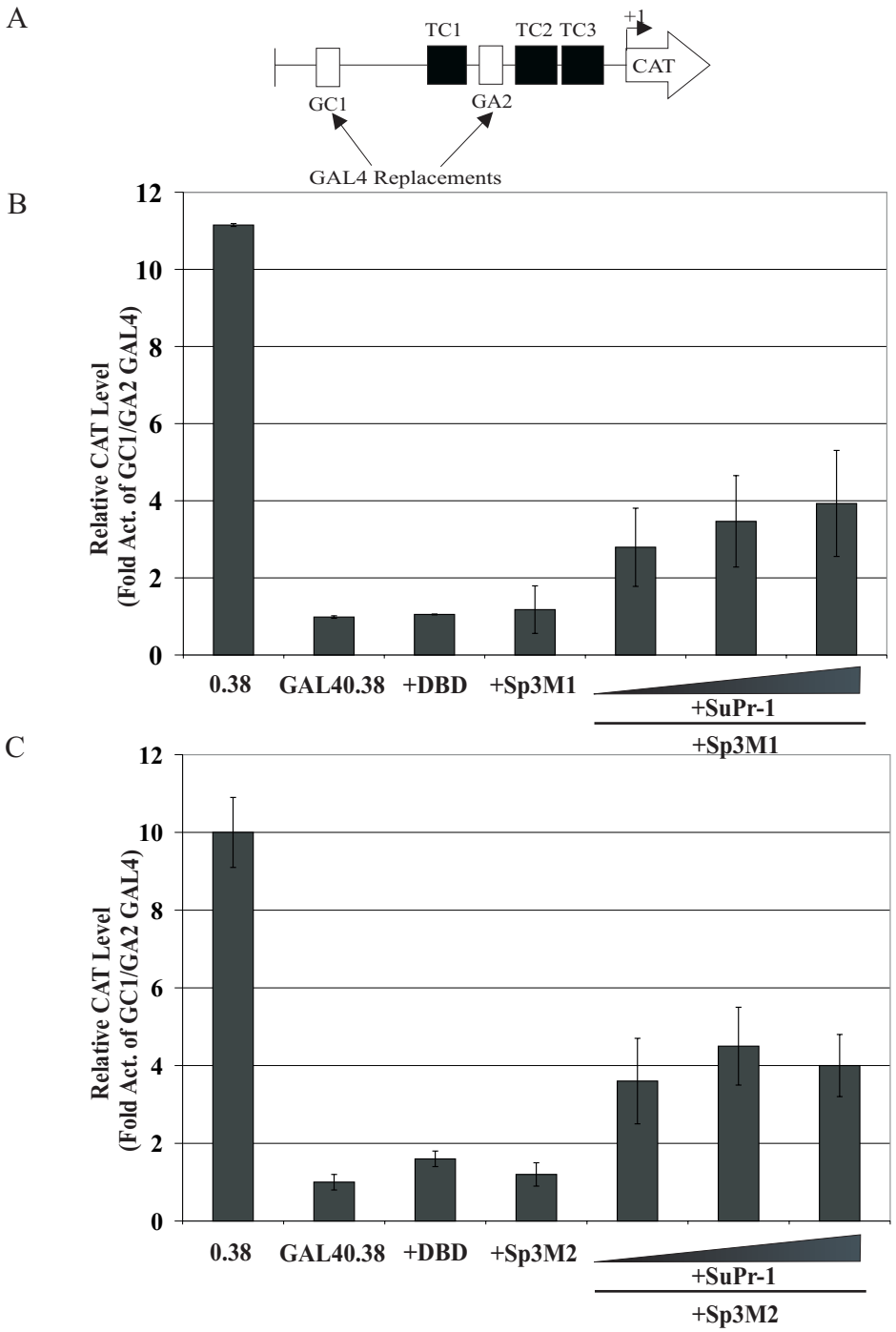


Figure 4.9. SUMOylation of the inhibitory domain of the short Sp3 isoforms prevents activation of SRC1A. (A) The wildtype 0.38SRC1A promoter was mutated to replace the endogenous Sp binding sites with GAL4 binding sites (SRC1AΔGC1/GA2-GAL4-CAT). (B) Activation of SRC1AΔGC1/GA2-GAL4-CAT reporter construct by GAL4Sp3M1 with and without co-transfection of SuPr-1 in HepG2 cells. (C) Activation of SRC1AΔGC1/GA2-GAL4-CAT reporter construct by GAL4Sp3M2 with and without co-transfection of SuPr-1 in HepG2 cells. The error bars are representative of the standard deviation between two experiments each performed in duplicate.

4.1.7. Discussion

4.1.7.1. Sp3 -mediated SRC1A Transactivation is Isoform -dependent

SRC is overexpressed in numerous cancer cell lines, including both colon cancer and hepatocellular carcinoma cell lines (Dehm and Bonham, 2004). In addition, SRC overexpression correlates strongly with increases in both c-Src protein and kinase activity. Furthermore, the increased levels of SRC mRNA transcripts have been shown to be the result of SRC transcriptional activation (Dehm, *et al*., 2001). These studies identify that the activation of the SRC gene in cancer cell lines is a legitimate contribution to increased c-Src kinase activity. Therefore, the mechanism of SRC transcriptional activation is a significant concern in discerning the role of c-Src deregulation in human cancer. To that end, the ubiquitously expressed SRC1A promoter has been characterized as being regulated by the Sp family of transcription factors and hnRNP K (Ritchie, *et al.*, 2000; Ritchie, *et al*., 2003).

Original analysis of the impact of the Sp family on SRC expression suggested that Sp1, but not Sp3, was an important mediator of SRC1A activity, however, these experiments were performed in SL2 cells with the pPacSp3M construct that expresses only the shortest Sp3 isoforms (Ritchie, *et al.*, 2000). In addition, recent discoveries prompted the re-examination of the effects -mediated by Sp3 with respect to SRC expression. For instance, until recently, an accurate clone of full length Sp3 was not available. The frequently cited, Sp3 clone that was formerly thought to be full length was not in reality a physiological form of Sp3, as a significant section was missing from the N-terminal region (Sapetschnig *et al*., 2004). In addition, the effects of Sp3 appeared to be promoter context -dependent; thus the actual role of a particular Sp3 isoform at a physiologically relevant promoter was impaired by the observations of other groups using artificial promoter systems and ill-defined Sp3 constructs. Finally, during the course of unrelated experiments, a member of the Bonham laboratory identified a potential role for Sp3 in SRC1A activation (Dehm, *et al*., 2004). Clearly, the capacity by which Sp3 exerts its effects on the SRC1A promoter needed to be re-evaluated.

To this end, a series of SRC reporter constructs were generated in which the Sp binding sites were replaced with GAL4 sequences, and a systematic examination of

each physiologically relevant isoform in mammalian cells was carried out. In addition, the N-terminally deleted form of Sp3, which had previously been thought to represent full-length Sp3 was examined. In parallel, a similar study in SL2 cells using the wild-type human promoter was carried out. From these experiments several significant observations were made. As expected, Sp1 was an impressive activator of the SRC1A promoter in both mammalian and insect cells. In contrast, whereas the physiologically relevant full length Sp3li was a modest activator of SRC1A, the two shortest Sp3 isoforms, Sp3M1/M2, were unable to activate the SRC1A promoter in either mammalian or insect cells. Significantly, the N-terminally truncated form of Sp3, Sp3ΔN, was able to activate the SRC1A promoter at least as effectively as Sp1 in all cell lines tested. Moreover, Sp3ΔN activated the SRC1A promoter 2-3 fold more effectively than Sp3li in mammalian cells and at least 5 fold more effectively in SL2 cells. These observations suggest that there may be a negative inhibitory region within the N-terminal region of Sp3.

The existence of a negative regulatory region within the N-terminus of Sp3 is quite plausible as the 100 residues missing from the N-terminal truncated form of Sp3 include hydrophobic residues, such as alanine and glycine-rich tracts, as well as several glutamine residues. These residues could mediate differences in the folding of full length Sp3 that might alter protein architecture and associations with co-activators to render the protein a far less potent activator. Similarly, the activation potential of Sp3 may also be hindered by a yet unidentified N-terminal post-translational modification. As discussed in the literature review, the phosphorylation of both Sp1 and Sp3 has alternate affects depending on the system being studied (Chu, *et al*., 2003: Arinze and Kawai, 2003). However, to date, nothing has been published suggesting that a discreet residue within the N-terminal domain of Sp3 is responsible for the disparities in activation potential between the true full length and truncated forms of Sp3. Aside from structural differences, the functional differences between Sp3li and Sp3ΔN are extremely important if the number of publications describing results obtained using the non-physiological Sp3ΔN are considered. Many groups, including the Bonham laboratory, used the non-physiological form of Sp3 or shorter forms of Sp3 in the

characterization of promoter regulation. As such, the true role of Sp3 in the transcriptional regulation of the SRC1A promoter is only now being revealed.

4.1.7.2. Transactivation of SRC1A by the Sp Family is Binding Site and Cell Line -dependent

Through the use of GAL4 reporter constructs, the effect of Sp1 and Sp3 at discreet SRC1A binding sites was measured. These results suggested that Sp1 was unable to activate the SRC1A promoter via the GC1 binding site, in HepG2 cells. Curiously, Sp1 was an activator of this site in SW480 cells, as well as in the insect cell line. Moreover, both of the longer Sp3 isoforms, Sp3li and Sp3ΔN, were able to activate SRC1A through the GC1 site in all cell lines analysed. Due to the utilization of GAL4 constructs, an important conclusion can be drawn from these observations. Since in the GAL4 system all of the fusion constructs contained the same GAL4 DNA binding domain, the inability of Sp1 to activate the GC1 site of SRC1A was not a result of impeded binding. As compromised binding of Sp1 to its DNA target is therefore ruled out as a potential deterrent to Sp1 -mediated SRC1A activation via this site, only a few alternatives remain. One possibility is that Sp1 -mediated activation could be compromised by a post-translational modification such as glycosylation and/or phosphorylation in this cell line (Li, *et al.*, 2004). However, although the selective modification of Sp1 is possible within a particular cell line, one would expect that Sp1 -mediated activation via the alternative site would also be precluded if this were indeed the case. The fact that SRC1A is still activated by Sp1 via the GA2 site in this cell line suggests that this possibility is unlikely. Another potential impairment to Sp1-mediated activation via the GC1 site is the distance of the GC1 site from the SRC1A core promoter region. It is possible that the association of Sp1 with critical co-activators is impeded within this cell line at this distance from the core promoter elements. In this scenario, Sp1 bound to the GA2 site may be sufficiently proximal to the core promoter elements that an interaction with supplementary factors is either facilitated or unnecessary. Furthermore, it is known that Sp1 forms homotetramers to amplify activation, as well as associates with the co-activator CRSP to enhance PIC assembly (Suske, 1999; Ryu, *et al*., 1999). Indeed, experiments with the human Ha-ras promoter have identified a key role for the most proximal Sp1 binding site in transcription start

site selection and enhanced activation, likely through interactions with co-activators (Lu, *et al*., 1994). This scenario is plausible considering that the activation of the SRC1A promoter via both GC1 and GA2 by Sp1 has the same fold value as activation through the GA2 site alone in HepG2 cells (~0.6), suggesting that the GC1 site is not contributing to enhanced SRC1A activation. Despite the unique perspective gained by the equalized binding of the GAL4 fusion constructs, further studies will be needed to elucidate the role of binding sites on Sp1-mediated activation of the SRC1A promoter in different cell lines.

4.1.7.3. SUMOylation of the Inhibitory Domain of Sp3 Isoforms Mediates Differential Activation of the SRC1A Promoter

The post-translational modification of regulatory proteins is an effective mechanism to rapidly and radically alter the effect of a protein in a particular system. Sp3 has been reported to be modified by both acetylation and SUMOylation (Braun, *et al.*, 2001; Ross, *et al.*, 2002; Ammanamanchi, *et al.*, 2003; Splenger, *et al.*, 2005). Though modification by either moiety is targeted to an identical lysine residue within the inhibitory domain of Sp3, the ramifications of each modification are remarkably opposite.

The acetylation of Sp3 is frequently associated with increased activational activity but has also been implicated in the repression of Sp3 -mediated activation. In a recent study, ceramide treatment of a human lung adenomacarcinoma cell line reduced Sp3 -mediated activation of the hTERT promoter through a decrease in the acetylation of Sp3 (Wooten and Ogretmen, 2006). Similarly, in MCF-7L breast cancer cells, Sp3 was shown to mediate the activation of the transforming growth factor-beta receptor type II (RII) promoter following acetylation induced by TSA treatment (Ammanamanchi, *et al*., 2003). Conversely, Sp3 acetylation prevented transcriptional activation of two artificial promoters in mammalian and insect cells (Braun, *et al.*, 2001) In contrast, the SUMOylation of the key lysine residue within the inhibitory domain of Sp3 is exclusively associated with reduced Sp3 -mediated activation. Two studies, published at approximately the same time, highlight the repression of two promoter systems by SUMOylation of Sp3 in both mammalian and insect cells (Ross, *et*

al., 2002; Sapetschnig, *et al.*, 2002). Another study, published after the isolation of the physiologically relevant full length Sp3 isoform, also found SUMOylation of Sp3 to be detrimental to Sp3 -mediated activation (Sapetschnig, *et al.,* 2004). Although the majority of studies suggest that acetylated Sp3 is a more potent activator of transcription, it is not known if acetylation itself plays a role in increasing Sp3 -mediated activation or if the prevention of SUMOylation, by acetylation, increases Sp3 -mediated activation. Clearly, there is a great deal of confusion surrounding the role of post-translational modifications of Sp3 in Sp3 activation or repression potential.

The finding that acetylation may impede the activation potential of Sp3 was particularly interesting within the context of SRC expression. A recurring topic throughout this thesis is the repression of SRC activity by HDIs. HDIs disrupt the balance between HAT and HDAC activity, ultimately tipping the scale towards increased acetylation of histones and other important factors. If HDI-mediated acetylation of Sp3 dampened the ability of Sp3 to activate the SRC1A promoter, this could be a possible mechanism by which HDIs repress SRC expression. Consistent with this idea, several groups have identified HDI-responsive Sp binding sites within the promoter regions of genes transcriptionally activated by HDIs (Xiao, *et al*., 2000; Ferguson, *et al*., 2003). Furthermore, at least one group has reported that HDI-mediated activation was associated with the acetylation of Sp1 followed by the recruitment of co-activators to the Sp1 binding site (Huang, *et al.*, 2005). As a logical extension of these studies, it is not difficult to imagine that acetylated Sp3 could recruit factors, in this case co-repressors, to the SRC1A promoter to hinder SRC expression, thereby providing a link between acetylation of Sp3 and repressed SRC1A activation. However, the mutation of the critical N-terminal lysine residue of Sp3li had little effect on Sp3li -mediated SRC1A activation in either mammalian or insect cells. Interestingly, in mammalian cells, mutating the critical lysine residue of Sp3ΔN resulted in a modest 2-fold increase in SRC activity. Moreover, upon treatment of mammalian cells with TSA, SRC1A activity was repressed, however, this repression was observed regardless of which Sp3ΔN construct was used. These results indicate that the modification of Sp3 by acetylation is not responsible for HDI-mediated SRC1A repression. Furthermore, the increased activation of SRC1A by the mutated Sp3ΔN was not observed in insect cells,

suggesting that the modest increase in Sp3ΔNK551R activation potential observed in mammalian cells is cell line specific.

The results obtained from the Sp3li and Sp3ΔN lysine to arginine and lysine to glutamine studies conflict with the observations of other groups that suggest that the prevention of SUMOylation, and in at least one case acetylation, dramatically increases the activation potential of Sp3. One possible explanation for this phenomenon may involve promoter context and the ability of Sp3 to modulate transcriptional activation. For example, initial studies of Sp3 SUMOylation utilized artificial or viral promoter systems and initial experiments were performed with Sp3ΔN. In these studies, Sp3ΔNK551R was an extremely strong activator of both the artificial G5-luc and BCAT-2 reporter constructs as compared to wild-type Sp3ΔN in both mammalian and insect cells (Ross, *et al.*, 2002; Sapetschnig, *et al.*, 2002). Furthermore, a more recent study suggested that the full length Sp3liK551R was a more potent activator of the artificial BCAT-2 promoter than wild-type full length Sp3 in insect cells. However, within this same study, in SL2 cells, Sp3liK551R managed only a modest 3 fold activation of the SV40-driven reporter construct, pGL3, as compared to wild-type (Sapetschnig, *et al.,* 2004). Interestingly, the sole investigation identifying Sp3 acetylation as a detriment to Sp3 activation potential also utilized the artificial BCAT-2 and SV40-promoter driven pGL3 (Braun, *et al.*, 2001). Taken together, these results suggest that the effect -mediated by Sp3 post-translational modifications may be promoter context -dependent.

Most surprisingly, the mutation of the critical lysine residue within the shortest inactive Sp3 isoforms, Sp3M1/M2, transformed these weak factors into potent activators of SRC expression in both insect and mammalian cell lines. Indeed, mutating the critical lysine residue to mimic an acetylated residue combined with SUMO protease 1 experiments confirms an important and significant role for SUMOylation in regulating the activity of Sp3M1/M2. The mechanisms of Sp3 regulation by SUMO-1 have not yet been elucidated, however, some suggest that SUMOylation prevents the association of the shortest Sp3 isoforms with co-activators, thus preventing Sp3M1/M2 -mediated activation (Splenger, *et al.*, 2005). SUMO-1 -mediated interruptions of Sp3 co-activator associations may not be as apparent with the longer Sp3 isoforms as they

contain two activation domains as opposed to the single activation domain adjacent to the inhibitory domain in Sp3M1/M2 (Fig. 3.1). As such, SUMOylation of these longer Sp3 isoforms would not abrogate activation to the degree observed with the shorter Sp3 isoforms. Similarly, SUMO-1 quenching may involve the recruitment of co-repressors, which would alter the assembly of transcriptional complexes at the core promoter regions and repress transcription. In agreement with this idea, SUMO-1 interacts with the CHD3/ZFH zinc-finger-containing helicase present in histone deacetylase complexes (Verger, *et al*., 2004). However, while this mechanism may occur with other factors it is unlikely that SUMO-1 -mediated Sp3M1/M2 silencing is a result of the recruitment of co-repressors. The recruitment of co-repressors by SUMO-1 to Sp3 would result in similar consequences regardless of which Sp3 isoform was bound. As a result, one would expect that the activity of all Sp3 isoforms would be affected, which is not the case. Finally, SUMOylation is reported as modifying the subcellular localization of transcription factors, such as p53, thus influencing their ability to activate or repress transcription (Verger, *et al*., 2004). Again, this scenario is unlikely in the case of Sp3. The conjugation of SUMO-1 to the critical lysine residue within Sp3 is a global phenomenon, as observed in Western blots performed with SL2 cell lysates. However, the effect -mediated by SUMO-1 is observed exclusively through the shortest Sp3 isoforms. Whatever the mechanism, SUMOylation significantly alters the ability of Sp3M1/M2 to enhance SRC1A activity.

4.1.7.4. Scope and Significance

The Sp family of transcription factors is relatively well characterized in their ability to modulate transcription. The opposing regulation of the SRC1A promoter by Sp1 and Sp3 has been previously described in both mammalian cancer cell lines and insect cell lines. However, recent developments concerning the multiplicitous nature of Sp3 have forced the re-evaluation of the role these factors exert within the context of the SRC1A promoter. The work described herein suggests that while the physiological isoforms of Sp3 (Sp3li) are modest SRC1A activators, the shortest isoforms (Sp3M1/M2) are incapable of activating SRC1A. However, although the ability of Sp3M1/M2 to activate SRC1A transcription is directly related to SUMOylation, SUMOylation of Sp3li had little effect on SRC1A activation. Interestingly, these results

suggest that activation by Sp3 is promoter context, binding site, cell line and modification -dependent, and therefore provide important clues in delineating the role of Sp1 and Sp3 in the regulation of SRC1A and other physiological promoters.

4.2. MODIFICATIONS AT SRC AND p21^{WAF1} PROMOTER LOCI IN RESPONSE TO HISTONE DEACETYLASE INHIBITORS

4.2.1. TSA Induced Acetylation Changes At SRC and p21^{WAF1} Promoters

The SRC1A and SRC1α promoters are both directly repressed by histone deacetylase inhibitors (HDIs), such as TSA and Sodium Butyrate. Previous work demonstrated that this repression did not require new protein synthesis and discrete HDI responsive elements could not be identified (Kostyniuk, *et al*., 2002; Dehm, *et al*., 2004). Historically, it was believed that HDIs act at the histone level to alter chromatin dynamics through the inactivation of HDACs to result in histone hyperacetylation and increased transcriptional activation (Santos-Rosa and Caldas, 2005). However, this model does not explain transcriptional repression of gene expression by these agents. Indeed, changes in the acetylation status of histones associated with genes repressed by HDAC inhibitors, such as SRC, have not been reported. Therefore, a systematic investigation of the changes in histone H3 and H4 acetylation status at the promoter regions of two genes differentially affected by HDAC inhibitors was carried out. For this study, the histones proximal to both SRC promoters were analysed, as they are both transcriptionally repressed by HDIs. In addition, the acetylation of histones proximal to the p21^{WAF1} promoter was also studied as p21^{WAF1} is transcriptionally activated by HDIs and should demonstrate high levels of histone acetylation (Rosato and Grant, 2003).

As transcriptional activation usually corresponds with increased histone acetylation, it would be expected that an increase in acetylation at the p21^{WAF1} promoter and a decrease in histone acetylation at the SRC promoter regions would be observed in response to TSA treatment. To test this model and identify changes in histone acetylation at promoter proximal regions, chromatin immunoprecipitation (ChIP) assays were performed with antibodies specific for histone H3 acetylated residues 9 and 14 (H3Ac9/14) and histone H4 acetylated residues 5, 8, 12 and 16 (H4Ac5/8/12/16). The acetylation of these residues was analysed, as histone acetylation of these residues is commonly associated with transcriptionally active genes (Santos-Rosa and Caldas, 2005). ChIPs were performed with HT29 cells that had been treated at various time points with 1μM TSA (Fig. 4.10). HT29 cells were used in this study due to previous findings from the Bonham laboratory suggesting that SRC transcription is initiated

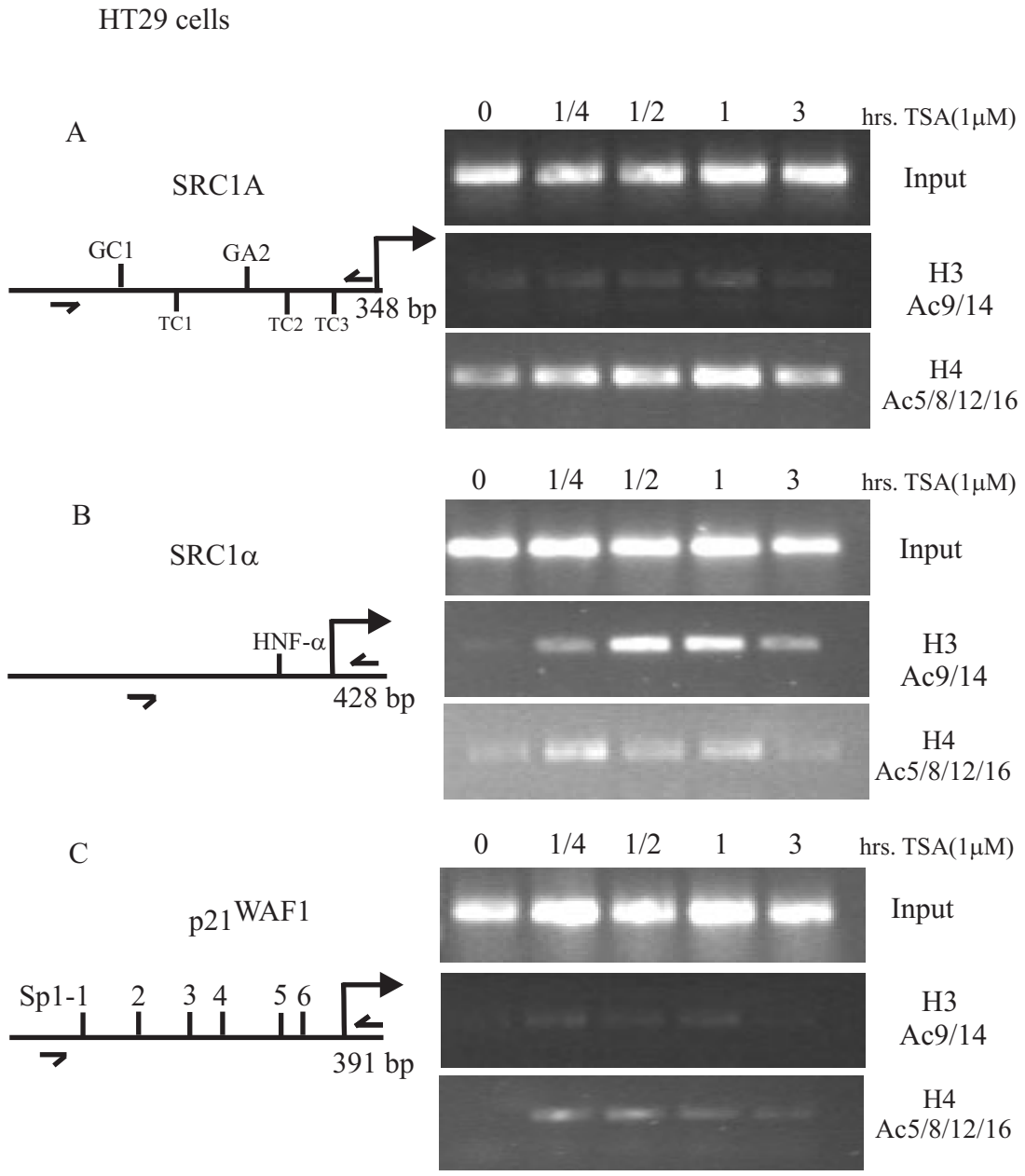


Figure 4.10. Effect of TSA on acetylation of Histone H3 and Histone H4 at the SRC and p21^{WAF1} promoters. ChIP assays were performed on HT29cells that had been treated for various time points with 1μM TSA. Proteins bound to DNA were cross-linked and immunoprecipitations were performed on cell lysates with antibodies specific to histone H3 acetylated lysines 9and 14, histone H4 lysines 5, 8, 12 and 16 and an antibody specific to integrin (negative control antibody, not shown). Following reversal of cross-links, immunoprecipitates underwent PCR with primers specific for the SRC1A (A), SRC1α (B) and p21^{WAF1} (C) promoters. These are representatives of experiments performed at least three times in each cell line.

equally from the SRC1A and SRC1α promoter regions in this cell line (Dehm, *et al*., 2001). Likewise, the $p21^{WAF1}$ promoter is also active in this cell line (Dehm, *et al*., 2004). PCR primers were designed that were specific for the SRC1A, SRC1α and $p21^{WAF1}$ promoter regions and used to amplify the DNA obtained from the ChIP assays (Fig. 4.10.). Interestingly, at all three promoter loci analysed, an increase in histone H3 and H4 acetylation was observed. It is important to note that although increases in SRC1α histone acetylation were more dramatic than that observed at the SRC1A promoter locus in HT29 cells, the pattern of change in histone acetylation was similar at both loci.

This experiment was repeated in HepG2 cells (Fig. 4.11.). This cell line was analysed as a result of previous studies suggesting, as with HT29 cells, SRC transcription is initiated equally from both the SRC1A and SRC1α promoter (Dehm, *et al*., 2001). Unfortunately, the changes in histone acetylation at the $p21^{WAF1}$ promoter was not analysed alongside the SRC promoters as previous studies have suggested that the increased $p21^{WAF1}$ expression -mediated by HDIs is not a result of transcriptional activation in this cell line (Hirsch and Bonham, 2004). Similar to that which was observed in HT29 cells, the SRC1A and SRC1α promoter regions displayed an enrichment of acetylated histone residues upon TSA treatment. However, in HepG2 cells the increase in TSA -mediated acetylation was dramatic and immediate at both histone H3 and H4 residues. Compared to HT29 cells, however, H3K9/14 acetylation increased strikingly at the SRC1α promoter and only slightly at the SRC1A promoter. Likewise, H4K5/8/12/16 acetylation increased in a less obvious fashion in HT29 cells than the increase observed in HepG2 cells. Overall, histone acetylation increases at the SRC promoter regions and $p21^{WAF1}$ promoter region in response to TSA treatment. Therefore, these results suggest that histone acetylation status may not be indicative of the transcriptional activity of a particular promoter in response to TSA treatment.

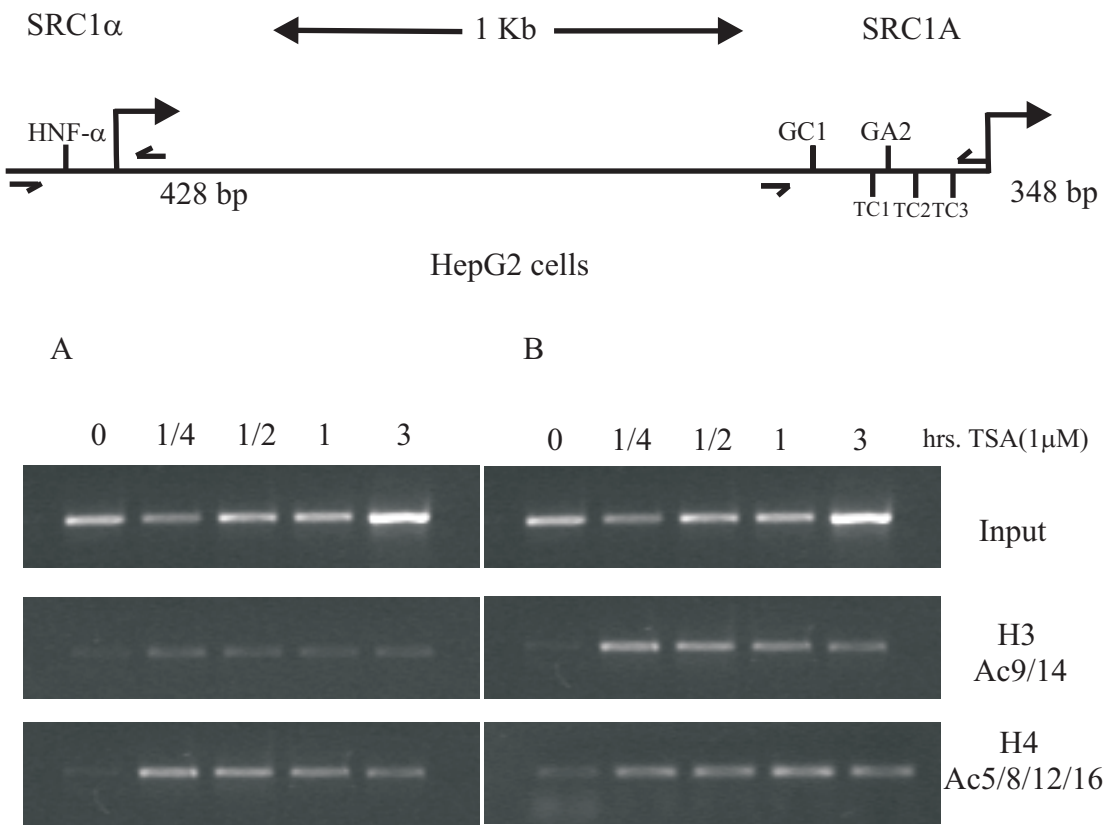


Figure 4.11. Effect of TSA on acetylation of Histones H3 and H4 at the SRC promoters. ChIP assays were performed on HepG2 cells that had been treated for various time points with 1μM TSA. Proteins bound to DNA were cross-linked and immunoprecipitations were performed on cell lysates with antibodies specific to histone H3 acetylated lysines 9and 14, histone H4 lysines 5, 8, 12 and 16 and an antibody specific to integrin (negative control antibody, not shown). Following reversal of cross-links, immunoprecipitates underwent PCR with primers specific for the SRC1α (A) and SRC1A (B) promoters. These are representatives of experiments performed at least three times in each cell line.

4.2.2. TSA -mediated Changes in Acetylation of Lysine Residues 9 and 14 on Histone H3

To further explore the results observed with histone H3Ac9/14 antibodies, a ChIP approach was again utilized to identify exactly what changes in acetylation were occurring at specific histone H3 lysine residues. This experiment was important because the histone H3Ac9/14 antibody can recognize acetylated H3K9 and/or acetylated H3K14. As a result, antibodies specific for acetylated H3K9 (H3Ac9) and acetylated H3K14 (H3Ac14) were obtained and ChIP assays were performed in HepG2 and HT29 cells treated with 1μM TSA. Primers specific for the SRC1A, SRC1α and $p21^{WAF1}$ amplicons were again used in these studies (Fig. 4.12). In HepG2 cells, TSA treatment resulted in an increase in H3K14 acetylation at both the SRC1A and SRC1α promoters. Conversely, TSA treatment caused a decrease in H3K9 acetylation at the SRC1A promoter and a very slight increase in acetylation at the SRC1α promoter. As before, the $p21^{WAF1}$ promoter region was not analysed in HepG2 cells. In HT29 cells, H3K9 acetylation increased at all promoter regions analyzed. However, while H3K9 acetylation at the $p21^{WAF1}$ promoter increased very gradually over the time course of TSA treatment, the increase in acetylation was more impressive at the SRC promoter regions. H3K9 acetylation is frequently associated with transcriptionally active chromatin regions, whereas H3K9 methylation is associated with transcriptionally silent heterochromatin regions (Eskeland, *et al*., 2007). As a result of the observed decrease in H3K9 acetylation at the SRC1A promoter in HepG2 cells, ChIPs were performed with an antibody specific to trimethylated H3K9. The purpose of this experiment was to observe if the decrease in H3K9 acetylation correlated with an increase of H3K9 trimethylation, which would signify a transition form a transcriptionally active chromatin region to a transcriptionally silent chromatin region. However, H3K9 trimethylation could not be seen at any point before or after TSA treatment suggesting that despite the loss of H3K9 acetylation, H3K9 trimethylation was not occurring (data not shown). Moreover, although TSA led to an increase in H3K14 acetylation at the SRC1A, SRC1α and $p21^{WAF1}$ promoters, the increase in acetylation at this residue was more dramatic at the $p21^{WAF1}$ promoter as compared with the SRC promoters. In summary, in HepG2 cells, the SRC1A promoter displayed a decrease in H3K9

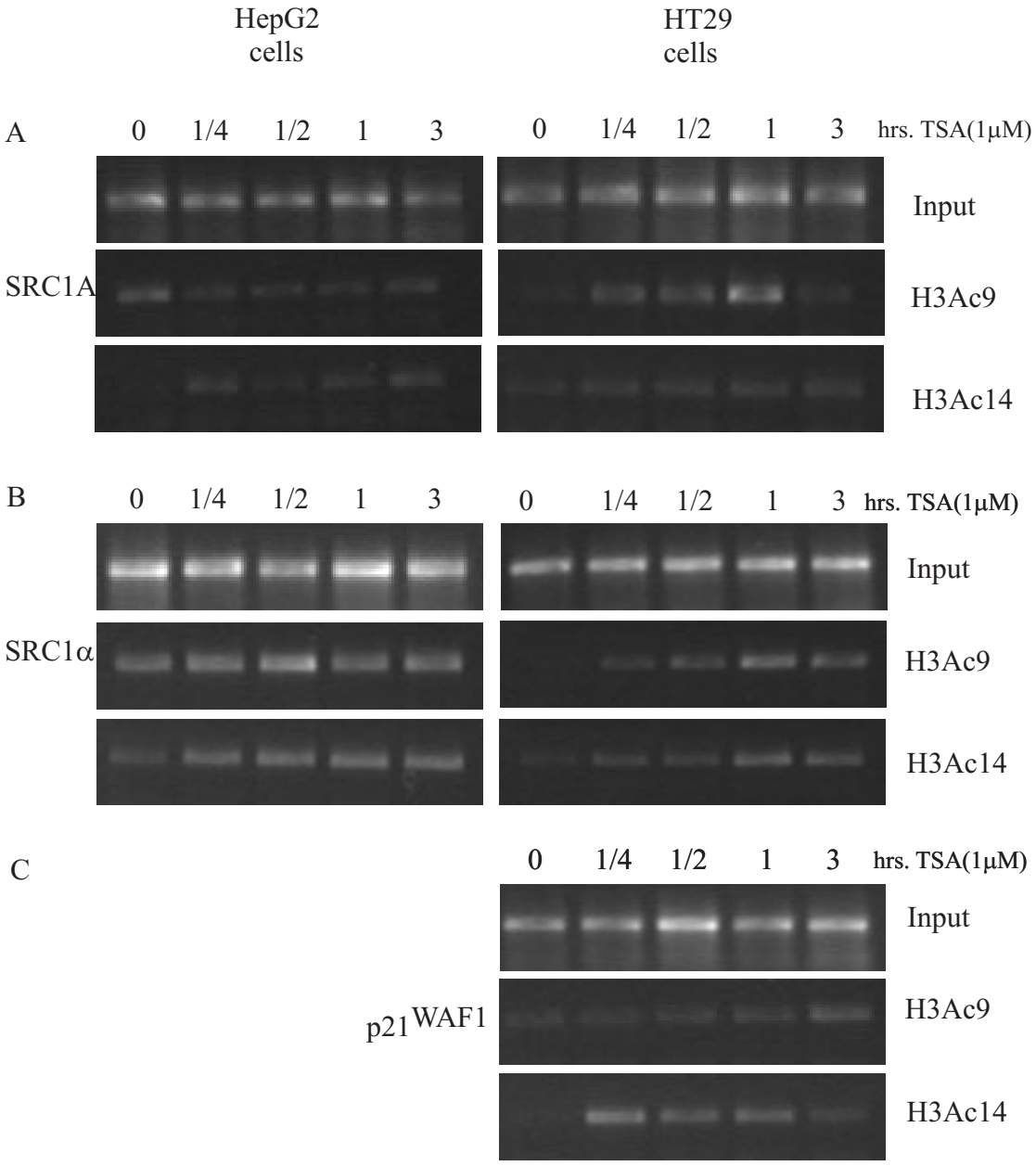


Figure 4.12. Effect of TSA on acetylation of Histone H3 lysines 9 and 14 at the SRC and p21^{WAF1} promoters. ChIP assays were performed on HepG2 and HT29 cells that had been treated for various time points with 1μM TSA. Proteins bound to DNA were cross-linked and immunoprecipitations were performed on cell lysates with antibodies specific to histone H3 acetylated lysine 9, histone H3 acetylated lysine 14 an antibody specific to integrin (negative control antibody, integrin). Following reversal of cross-links, immunoprecipitates underwent PCR with primers specific for the SRC1A(A), SRC1α (B) and p21 (C) promoters. These are representatives of experiments performed at least three times in each cell line.

acetylation but an increase in H3K14 acetylation upon TSA treatment, whereas, the SRC1α promoter showed an increase in acetylation at both H3 lysine residues. In HT29 cells, all three promoter regions analyzed demonstrated increased H3K9 and H3K14 acetylation in response to TSA. With the exception of decreased H3K9 acetylation at the SRC1A promoter in HepG2 cells, these findings correlate well with the results obtained with ChIP assays using the pan acetylated histone H3 antibodies.

4.2.3. TSA -mediated Changes in Acetylation of Lysine Residues 5, 8, 12 and 16 on Histone H4

Similar to the histone H3Ac9/14 antibody (4.2.2.), the histone H4Ac5/8/12/16 antibody was capable of recognizing the epitope of any or all of the pertinent histone H4 acetylated lysine residues (K5, 8, 12, 16). In order to fully characterize the impact of TSA on the acetylation of histone H4 lysine residues, individual ChIP assays were necessary with antibodies specific for the acetylated form of each of the lysine residues (Fig.4.13.). In HepG2 cells, SRC1A and SRC1α promoters showed increased histone H4K12 and histone H4K16 acetylation upon TSA treatment. However, it should be noted that acetylation of these histone residues at the SRC1α promoter was marginal and, therefore, difficult to detect. Histone H4K8 acetylation either increased slightly or remained unchanged at both SRC promoter regions in response to TSA. Similarly, histone H4K5 acetylation increased very slightly or not at all at the SRC1A promoter, however, an increase in acetylation at H4K5 was observed at the SRC1α region.

In HT29 cells, histone H4K5 acetylation increased at the p21 and SRC promoter regions in response to TSA treatment. However, enrichment of histone H4K5 acetylation was marginal at the SRC1A promoter as compared to the other two regions analyzed. Histone H4K8 acetylation decreased at all three promoter regions studied. Histone H4K12 acetylation increased slightly at the SRC1A promoter but decreased at the SRC1α and p21 promoter regions. Conversely, histone H4K16 acetylation decreased at the SRC1A promoter but increased slightly at the SRC1α and p21 promoters.

Overall, these observations correlate well with the pan acetylated histone H4 ChIP data. In HepG2 cells, at residues where there was a change in acetylation, there

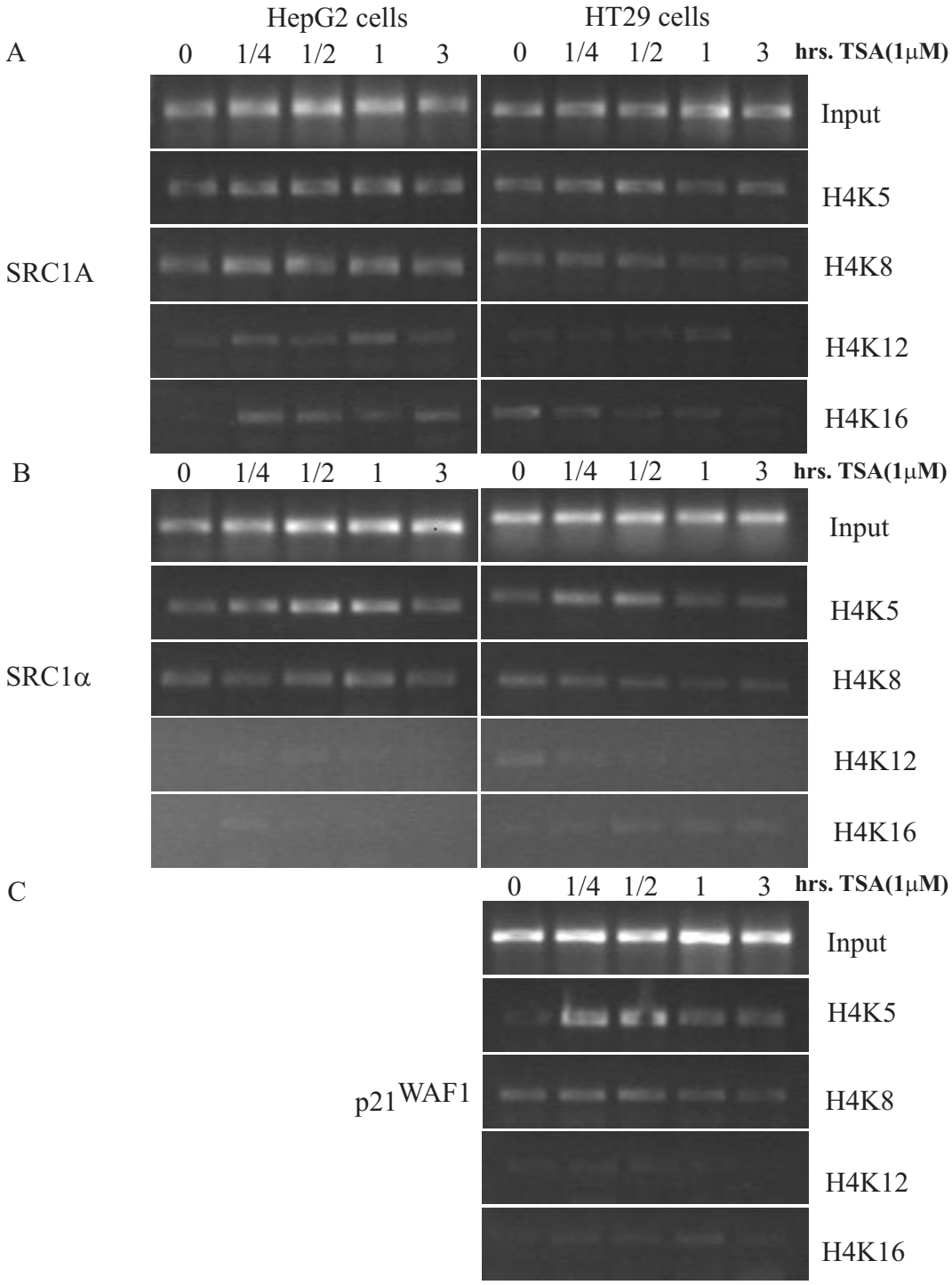


Figure 4.13. Effect of TSA on acetylation of Histone H4 lysine 5, 8, 12 and 16 at the SRC and p21^{WAF1} promoters. ChIP assays were performed on HepG2 and HT29 cells treated for various time points with 1μM TSA. Proteins bound to DNA were cross-linked and immunoprecipitations were performed on cell lysates with antibodies specific to histone H4 acetylated lysine residues 5, 8, 12 and 16. Following reversal of cross-links, immunoprecipitates underwent PCR with primers specific for the SRC1A (A), SRC1α (B) and p21^{WAF1} (C) promoters. These are representatives of experiments performed at least three times in each cell line.

was an increase in acetylation upon TSA treatment. In HT29 cells, at each promoter studied, at least one residue displayed a dramatic increase in acetylation in response to TSA. Due to the ambiguity of antibody/epitope binding of the pan acetylated histone H3 and H4 antibodies, it was difficult to identify what epitope the antibody was recognizing and these studies aided in identifying the histone lysine residues affected by HDI treatment.

4.2.4. Trends in TSA -mediated Changes in Histone Acetylation at the SRC and $p21^{WAF1}$ Promoters

In order to gain an appreciation for the global changes in histone acetylation occurring at these promoter regions in response to TSA, a summary chart has been included (Table 4.1.). In general, the three promoter regions appear to be very similar in the promoter specific changes in acetylation. An obvious difference in HepG2 treated cells is that H3K9 is deacetylated at the SRC1A promoter but increased acetylation is observed at the SRC1α promoter. An important observation in HT29 cells, is that the SRC1α and $p21^{WAF1}$ promoter regions demonstrated greater similarity in histone acetylation changes as compared to the SRC1A promoter. This is of particular interest considering that both SRC promoters are transcriptionally repressed by HDIs whereas $p21^{WAF1}$ is transcriptionally activated by HDIs. These findings again contribute to the idea that the histone acetylation pattern is not an accurate predictor of promoter activity in response to TSA treatment.

4.2.5. Transcription Factor Occupancy on the SRC1A Promoter in Response to TSA Treatment

Previous experiments failed to identify discrete HDI response elements at either of the SRC1A or SRC1α promoters (Dehm, *et al*., 2004). In addition, the acetylation of histone residues at SRC promoter proximal regions was not indicative of HDI -mediated transcriptional activation or repression. As a result of these findings, the binding of gene-specific transcription factors, as well as an essential component of the pre-initiation complex (PIC) were analysed. It would be expected that if HDIs were indeed detrimental to the binding of activators, there would be a decrease in promoter

	HT29 cells			HepG2 cells	
	SRC1A	SRC1α	$p21^{WAF1}$	SRC1A	SRC1α
H3panAc	+	+	+	+	+
H4panAc	+	+	+	+	+
H3K9	+	+	+	—	+
H3K14	+	+	+	+	+
H4K5	+	+	+	O/+	+
H4K8	—	—	—	O/+	O/+
H4K12	+	—	—	+	+
H4K16	—	+	+	+	+

Table 4.1. Summary chart of acetylation specific ChIP analysis results. Promoter specific increased histone acetylation during the time course is indicated by the (+) symbol. Promoter specific decreased histone acetylation during the time course is indicated by the (—) symbol. No change or a marginal increase in histone acetylation is indicated by the (O/+) symbol.

occupancy of these factors upon TSA treatment. Therefore, to identify if promoter occupancy of known SRC1A activators was abrogated by TSA treatment, thereby impeding SRC activity, ChIP assays were performed.

Previous results (Fig. 4.1) suggested that in untreated HT29 cells Sp1 is bound to the SRC1A promoter. Similarly, we used HT29 and HepG2 cells that had been treated at various time points with TSA and performed ChIP analysis with antibodies specific for Sp1 (Fig. 4.14.). PCR was performed with primers specific for the SRC1A promoter and it was found that Sp1 remained at the SRC1A promoter upon treatment with TSA. One problem with these particular Sp1 studies was that the antibody used in these experiments was not from the same lot that was used to previously identify the presence of Sp1 at the SRC1A promoter (Fig. 4.1). As a result, the positive antibody bands were very faint and difficult to interpret. To confirm these PCR results, real-time PCR with taqman probes was implemented to identify fold changes in Sp1 occupancy at the SRC promoters with ChIP samples derived from TSA treated HT29 cells. The benefit of adopting a real-time PCR strategy in the analysis of transcription factor binding to the SRC1A promoter is due to the quantitative nature of real-time PCR. Other ChIP results were obtained through the use of a thermocycler and analysed by agarose gel electrophoresis. Unfortunately, this method is semi-quantitative at best as often many cycles are necessary to identify DNA enriched in the positive antibody samples, as high number of PCR cycles can result in less accurate results and the linear range of product amplification may be exceeded due to limiting reagents. Real-time PCR overcomes this obstacle by analysing the product after every cycle, thus allowing for quantitative analysis within the linear range of amplification. To analyze the Taqman results, values were determined by standardizing the samples to input C(T), converting C(T) values into DNA concentrations and then graphing as fold changes between the negative and positive antibody samples. This method resulted in the fold enrichment of Sp1 occupancy at the SRC promoters as compared to the negative control antibody, integrin. These results suggest that there is approximately a 30 fold difference in Sp1 occupancy at the SRC1A promoter as compared to the negative antibody and this enrichment did not change over the three hour time course (Fig.4.14. B.). As an

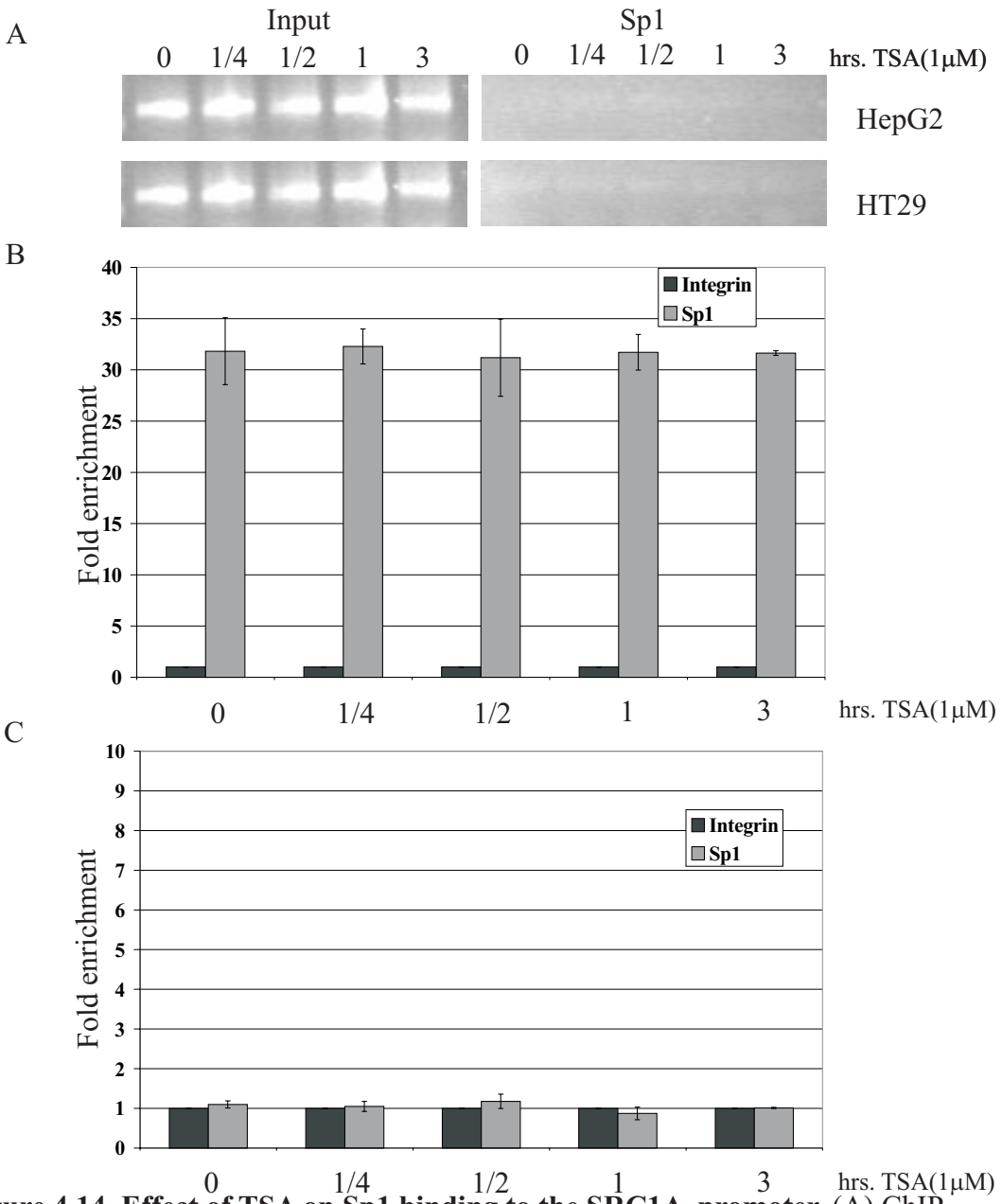


Figure 4.14. Effect of TSA on Sp1 binding to the SRC1A promoter. (A) ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific to Sp1. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for the SRC1A promoter. Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line. (B)Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1A promoter. (C) Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1α promoter. Both B and C values were determined by standardizing the samples to input C(T), converting C(T) values into DNA concentrations and then graphing as fold changes between the negative and positive antibody samples.

additional control, real-time PCR was performed with a probe and primers specific to the SRC1α promoter, which does not bind Sp1 (Fig. 4.14. C). As expected, these results showed no difference between Sp1 and integrin occupancy at this region, thus confirming the specificity of the SRC1A findings.

Similar experiments were performed to identify if Sp3 binding to SRC1A was affected by TSA treatment. ChIP assays with antibodies specific to Sp3 were performed with TSA treated HT29 and HepG2 cells. Again, the lot of Sp3 antibody that had been used in previous studies (Fig. 4.1.) could not be purchased and the results obtained from traditional PCR were difficult to interpret (Fig. 4.15. A). Real-time PCR, with a probe and primers specific to SRC1A, demonstrated that Sp3 was present at approximately a 10 fold enrichment as compared to the negative control antibody (Fig. 4.15. B). Significantly, these results demonstrated that Sp3 occupancy at the SRC1A promoter was not changed upon TSA treatment. As with the Sp1 studies, the SRC1α promoter region was used as a ChIP assay specificity control and again it was found that Sp3 does not bind to this region of the SRC locus (Fig. 14.5. C).

Taken together, these results show that Sp1 and Sp3 continue to bind the SRC1A promoter despite TSA treatment. This suggests that the effects exerted by TSA are not -mediated by either of these factors at the SRC1A promoter in either HepG2 or HT29 cells. Notably, promoter occupancy studies of the SRC1α specific transcription factor, HNF-1α, were not performed. Unfortunately, a HNF-1α specific antibody amenable to ChIP analysis was unavailable and as such the effect of TSA treatment on the HNF-1α promoter occupancy could not be measured by ChIP analysis.

4.2.6. RNA Polymerase II Occupancy on SRC Gene in Response to TSA Treatment

The binding of Sp1 and Sp3 to proximal promoter elements was not affected by TSA treatment. Furthermore, studies have shown that HDIs can attenuate transcriptional initiation by preventing the binding of basal transcription factors and RNA polymerase II (Svejstrup, 2004). In keeping with the model whereby HDIs mediate SRC repression through the abrogated binding of key factors to the SRC promoter regions, the occupancy of RNA polymerase II (RNA Pol. II) at the SRC core promoter regions in

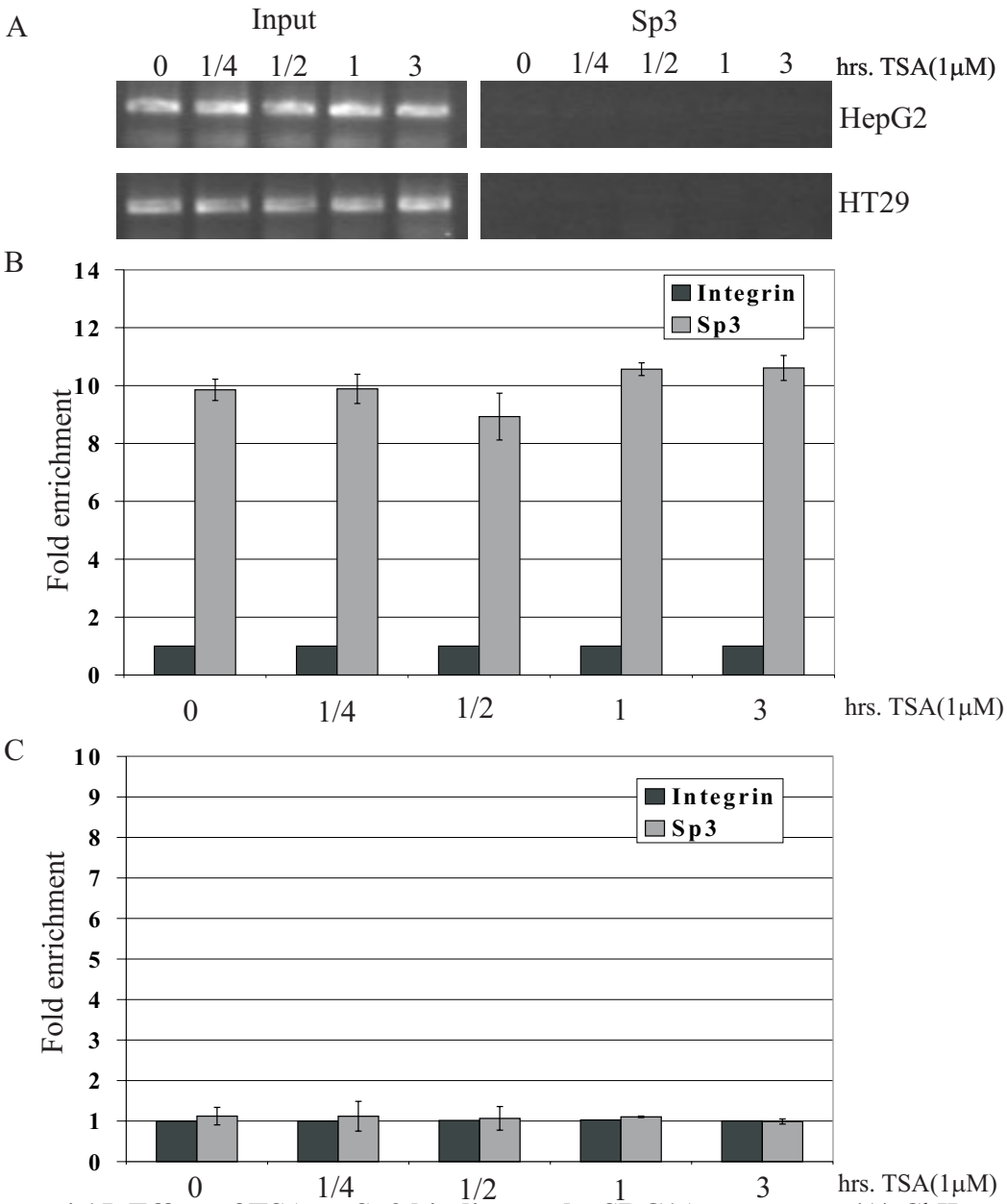


Figure 4.15. Effect of TSA on Sp3 binding to the SRC1A promoter. (A) ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific to Sp3. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for the SRC1A promoter. Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line.(B)Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1A promoter. (C) Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1α promoter. Both B and C values were determined by standardizing the samples to input C(T), converting C(T) values into DNA concentrations and then graphing as fold changes between the negative and positive antibody samples.

response to TSA treatment was analysed. If TSA treatment modifies RNA Pol. II binding to the SRC core promoter regions, SRC transcriptional activation would be severely crippled. Therefore, ChIP analysis was performed with an antibody specific for RNA Pol. II with HepG2 and HT29 cells treated for various times with TSA (Fig. 4.16.).

RNA Pol. II occupancy was not affected at the SRC1A and SRC1α promoters following TSA treatment. Therefore TSA -mediated SRC repression is not a result of the absence of this essential component of transcription. However, despite the presence of this factor at the promoters, the possibility of faulty transcriptional initiation and/or elongation could not be discounted. To address this issue, PCR was performed with the RNA Pol. II ChIP samples from the TSA treated cells using primers specific to several regions downstream of the SRC promoters. The first two regions analysed for changes in RNA Pol. II occupancy were regions between the SRC1A exon and SRC1B exon and a region between SRC1B and SRC1C exons (Fig. 4.17. A.). The times above the stars in the figure give the range of time necessary for the effects of TSA on RNA Pol. II entrapment at the promoter regions to be identified. The times were calculated assuming that RNA Pol. II transcribes DNA at a rate of 1-1.5 Kb per minute (Neugebauer, 2002; Mason and Struhl, 2005). PCR of the first amplicon did not yield any information to suggest that RNA Pol. II was ever bound to this region, before or after treatment (Fig. 4.17. B). Amplification of the genomic region between exons 1B/1C suggested that RNA Pol. II continued to be associated with this region following TSA treatment. Notably, amplification of this region was not as efficient as that of either of the promoter regions and the occupancy of RNA Pol. II at this region could be changing.

The next SRC genomic amplicons analyzed included two regions spanned by exons 4 and 5 and the 3' untranslated region (3'UTR) (Fig. 4.18. A). Importantly, the first exon 4/5 amplicon showed a definite decrease in RNA Pol. association after approximately 30 minutes, with detection being completely abrogated by the three hours time point (Fig. 4.18. B.). This phenomenon was especially apparent in HT29 TSA treated samples. Significantly, 30-45 minutes is the expected amount of time that it would take to actually be able to record a loss in RNA Pol. II at this region of the SRC

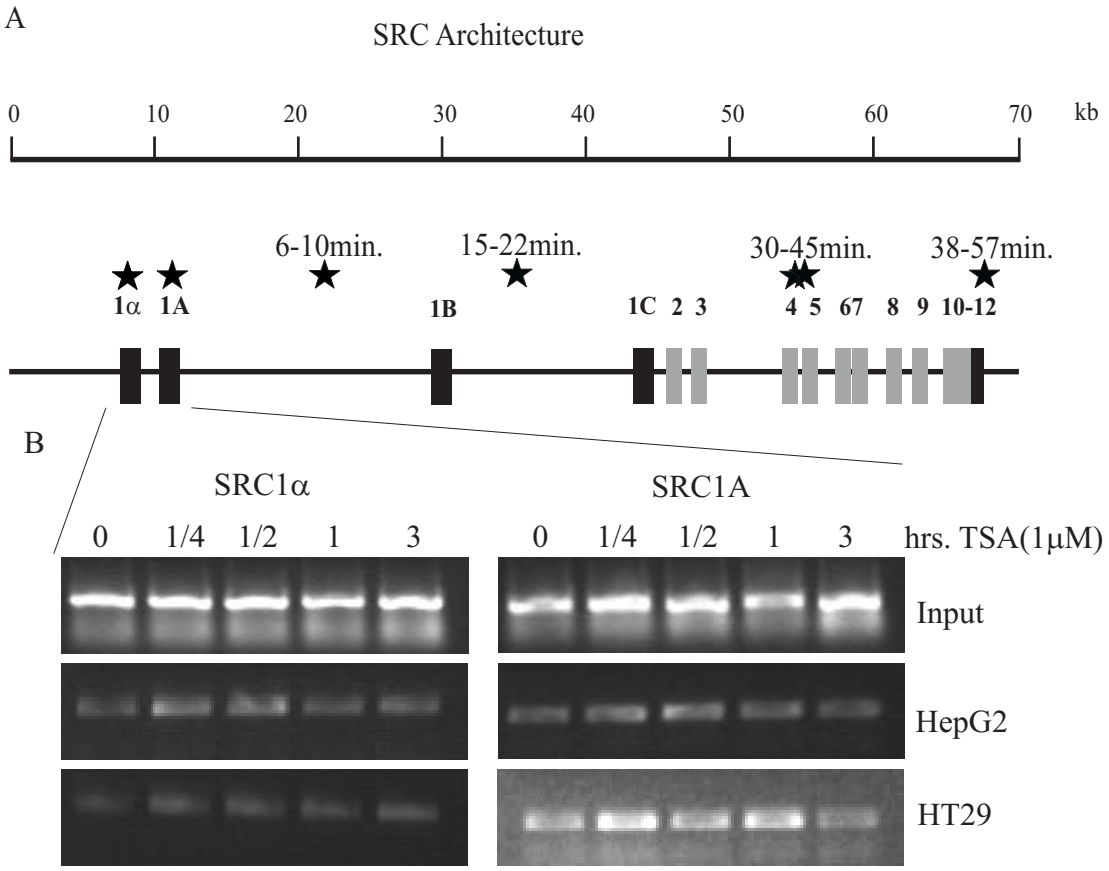


Figure 4.16. RNA polymerase II occupancy at the SRC promoters is not affected by TSA treatement. (A) SRC genomic architecture. Dark boxes indicate in the SRC untranslated regions and lighter boxes indicate exons that are translated. The time required for RNA polymerase II transcription from either the SRC1A or SRC1α promoters to the amplicon is indicated above the star. Time required was determined assuming a polymerase II rate of transcription of 1 or 1.5 Kb/minute. (B) ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific for RNA polymerase II. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for SRC1α and SRC1A promoters. Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line.

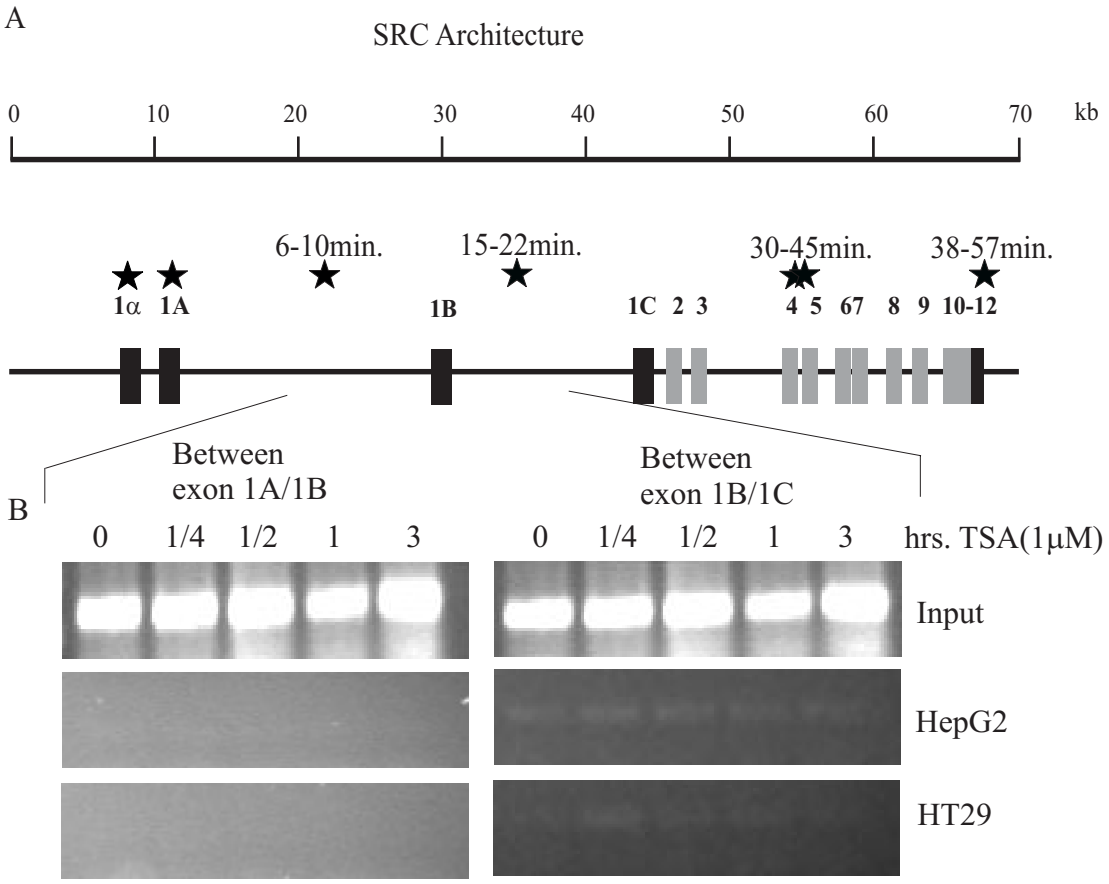


Figure 4.17. RNA polymerase II occupancy at SRC exons is not affected by TSA treatement. (A) SRC genomic architecture. Dark boxes indicate in the SRC untranslated regions and lighter boxes indicate exons that are translated. The stars indicate the regions amplified in the ChIP analysis. The time required for RNA polymerase II transcription from either the SRC1A or SRC1α promoters to the amplicon is indicated above the star. Time required was determined assuming a polymerase II rate of transcription of 1 or 1.5 Kb/minute. (B) ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific for RNA polymerase II. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for the regions between exon 1A/1B and exons 1B/1C. Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line.

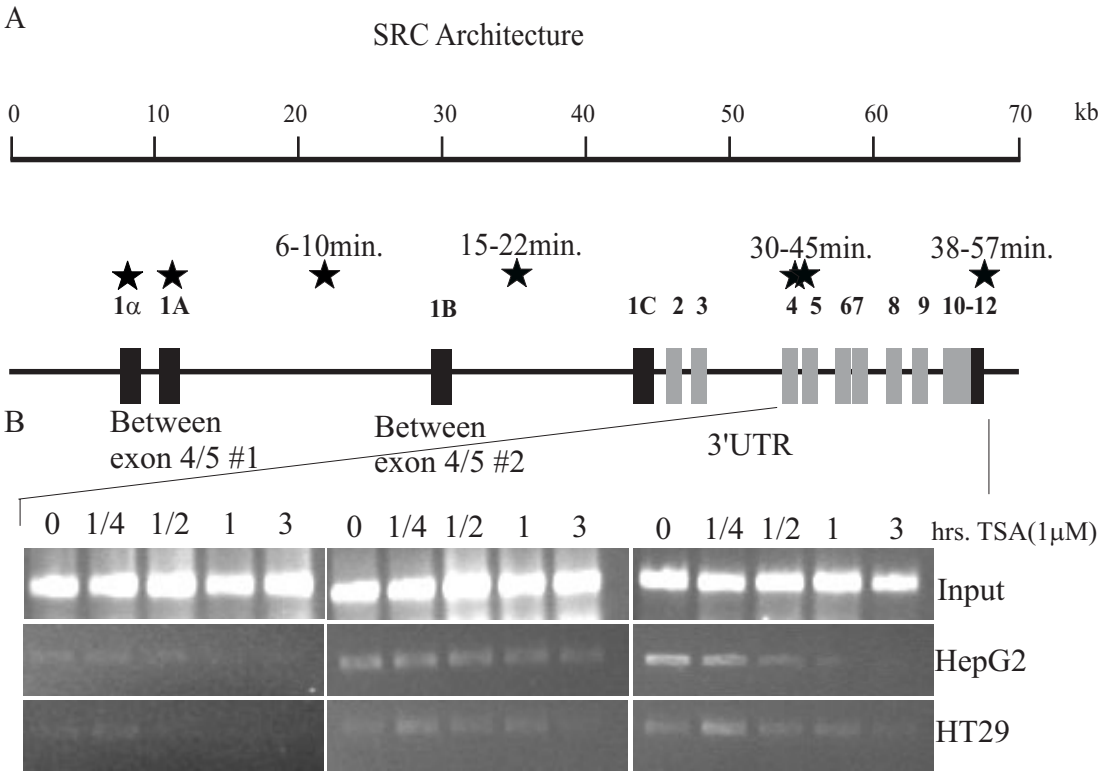


Figure 4.18. RNA polymerase II occupancy at the SRC 3'UTR is affected by TSA treatement. (A) SRC genomic architecture. Dark boxes indicate in the SRC untranslated regions and lighter boxes indicate exons that are translated. The stars indicate the regions amplified in the ChIP analysis. The time required for RNA polymerase II transcription from either the SRC1A or SRC1α promoters to the amplicon is indicated above the star. Time required was determined assuming a polymerase II rate of transcription of 1 or 1.5 Kb/minute. (B) ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific for RNA polymerase II. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for two regions between exons 4/5 and the 3' untranslated region (3' UTR). Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line.

gene if RNA Pol. II was sequestered at the promoter regions upon treatment. Conversely, the second amplicon between exons 4 and 5 showed continued detection of RNA Pol. II by the three hours time point, despite a subtle decrease in overall occupancy. Finally, there was an obvious loss of RNA Pol. II at the 3'UTR by the 30 minutes time point with an almost complete loss of Pol. II by the three hours time point in both cell lines studied.

In summary, RNA Pol. II remains present at both SRC promoters in HepG2 and HT29 cells after TSA treatment. A definite decrease in RNA Pol. II occupancy at the 3'UTR was observed by the 30 minutes time point, with an almost complete loss observed by three hours post treatment. These results suggest that RNA Pol. II may be confined to the promoter regions in response to TSA treatment.

4.2.7. Phosphorylation of RNA Polymerase II on SRC Gene in Response to TSA Treatment

An RNA Pol. II signal was present at the SRC promoter regions and yet was lost at regions downstream in response to TSA treatment. RNA Pol. II function is dictated by the phosphorylation of two key residues within its carboxy-terminal domain (CTD) (Meinhart, *et al*., 2005). The Human RNA Pol. II CTD is characterized as having 52 heptad repeats, with the phosphorylation of the second and fifth serine residues being key in RNA Pol. II function (Phatnani and Greenleaf, 2006). RNA Pol. II is characteristically phosphorylated at the fifth serine residue of the CTD heptad when RNA Pol. II is within the 5' region of a gene (Phatnani and Greenleaf, 2006). Typically, the phosphorylation of serine five decreases as the polymerase transverses the gene. The RNA Pol. II antibody used in the previous experiments was not phospho-specific. Therefore, to identify if TSA induced changes may occur via RNA Pol. II at the promoter regions, changes in the phosphorylation of both residues in response to TSA were addressed.

To identify if aberrant CTD phosphorylation was occurring in response to TSA, ChIPs were performed with an antibody specific to RNA Pol. II CTD phosphorylated serine 5 (RNA Pol. II (S5)) with TSA treated HeG2 and HT29 cells (4.19.). S5 of RNA Pol. II CTD was phosphorylated at the promoter regions of SRC. This phosphorylated

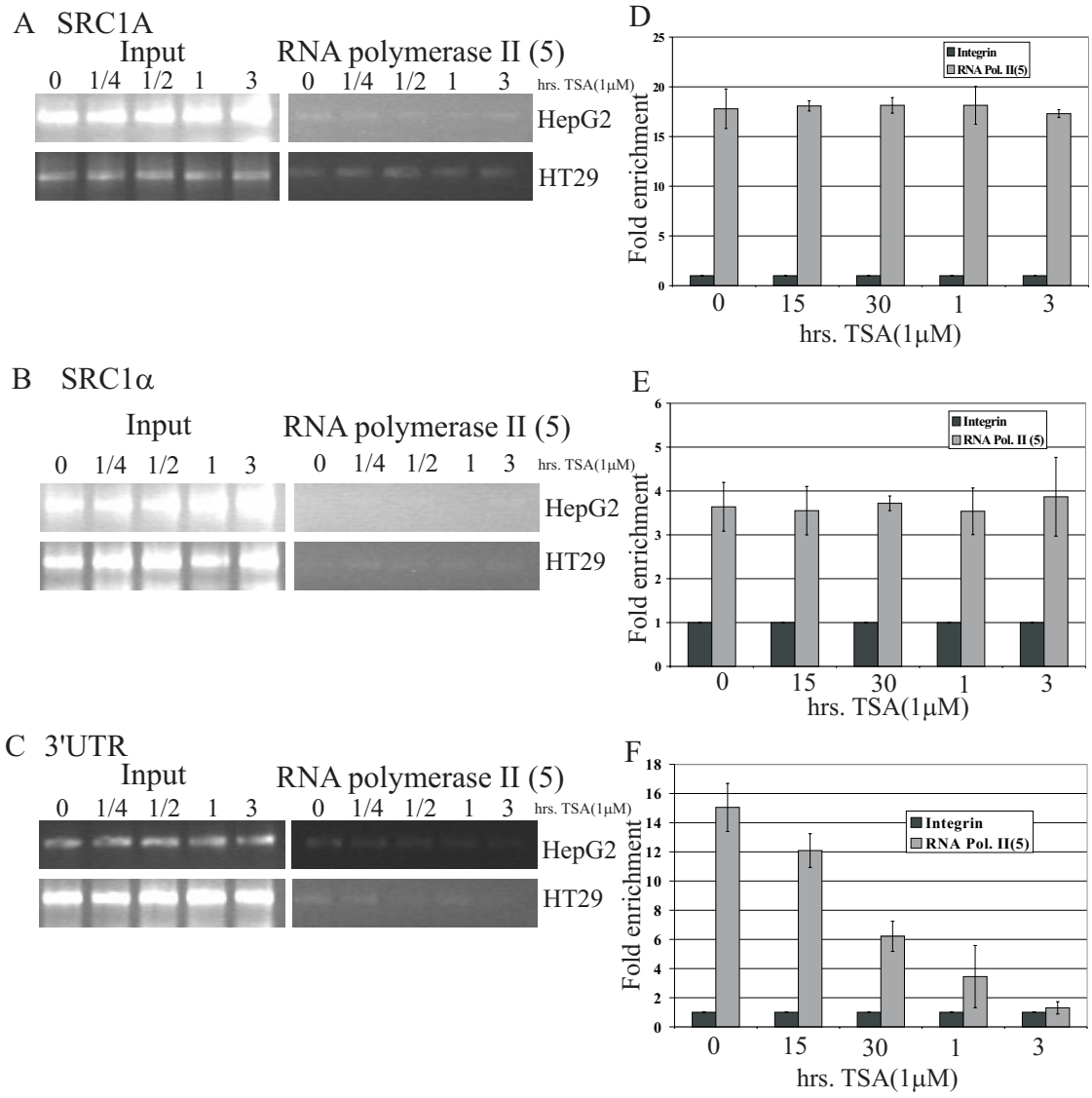


Figure 4.19. Effect of TSA on the serine 5 phosphorylated form of RNA Polymerase II binding to the SRC promoters and SRC 3'UTR. ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific to the serine 5 phosphorylated form of RNA Polymerase II. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for the SRC1A promoter (A), SRC1α promoter (B) or SRC 3'UTR (C). Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line. Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1A promoter (D), SRC1α promoter (E) and 3'UTR (F).Both B and C values were determined by standardizing the samples to input C(T), converting C(T) values into DNA concentrations and then graphing as fold changes between the negative and positive antibody samples.

form of Pol. II was maintained at the promoter regions despite TSA treatment (Fig.4.19. A, B.). Conversely, S5 CTD phosphorylation appeared to decrease at the 3' UTR of SRC in response to TSA (Fig. 4.19. C.). This observation was in agreement with previous RNA Pol. II results, whereas an overall decrease in RNA Pol. II occupancy at this locus had been previously observed (Fig.4.18.). These experiments were supported by real-time PCR data performed with samples from HT29 cells and graphed as indicated in a previous section (Fig. 4.19. D, E, F.). The real-time data suggests that there is a greater population of serine 5 phosphorylated RNA Pol. II at the SRC1A promoter as compared to the SRC1α promoter (nearly five fold difference in occupancy between the two promoters). Although RNA Pol. II (S5) is not commonly associated with the 3' distal regions of a gene, there was approximately a 14 fold enrichment at this region in untreated cells that rapidly decreased upon TSA treatment as compared to the negative control (α-integrin).

Conversely, the serine 2 phosphorylated CTD form of RNA Pol. II (RNA Pol. II (S2)) is under-represented at the promoter and is commonly most abundant at regions downstream of the promoter (Phatnani and Greenleaf, 2006). To identify where this form of RNA Pol. II was most abundant along the SRC gene, we performed ChIP with an antibody specific for RNA Pol. II (S2) in TSA treated HT29 and HepG2 cells (Fig. 4.20.). Interestingly, RNA Pol. II (S2) could be detected at both SRC promoter regions and these levels did not change upon TSA treatment. However, real-time results indicate that though RNA Pol. II (S2) can be found at the SRC promoter regions, it is at very low levels as compared to RNA Pol II (S5) enrichment at these regions. Whereby, RNA Pol. (S2) can only be found at two-fold the levels of the negative control antibody at the SRC1α promoter and four-fold the levels of the negative control at the SRC1A promoter. These levels were dramatically lower than what was observed with the antibody specific for RNA Pol. II (S5) at the SRC1α and SRC1A promoter regions (4 and 17-fold, respectively). In contrast, RNA Pol. II (S2) occupation at the 3'UTR is significantly higher than what was observed with the RNA Pol. II (S5) experiments (40-fold versus 17-fold enrichment) but the presence of this phospho-specific RNA Pol. II moiety also decreased in response to TSA.

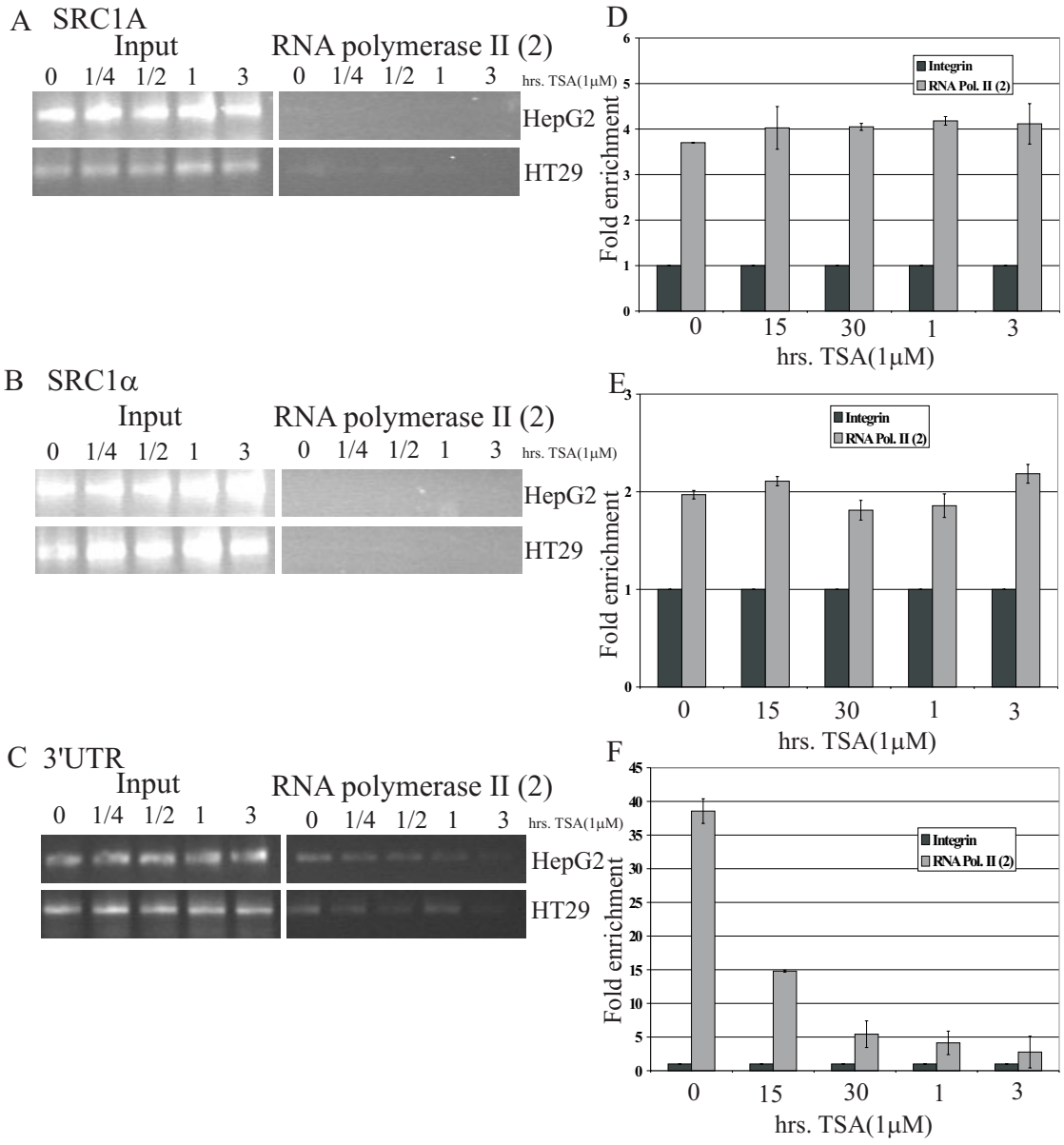


Figure 4.20. Effect of TSA on the serine 2 phosphorylated form of RNA Polymerase II binding to the SRC promoters and SRC 3'UTR. ChIP assays were performed on HepG2 and HT29 cells, treated at various time points with TSA, with an antibody specific to the serine 2 phosphorylated form of RNA Polymerase II. Following immunoprecipitation and de-crosslinking, DNA was amplified using primers specific for the SRC1A promoter (A), SRC1α promoter (B) or SRC 3'UTR (C). Samples were resolved on an agarose gel. The input samples are non-immunoprecipitated samples. The negative antibody control (α-Integrin) samples are not shown. These results are representative of three independent experiments performed in each cell line. Real-time PCR of two averaged HT29 ChIP assays performed with probes and primers specific for the SRC1A promoter (D), SRC1α promoter (E) and 3'UTR (F). Both B and C values were determined by standardizing the samples to input C(T), converting C(T) values into DNA concentrations and then graphing as fold changes between the negative and positive antibody samples.

Taken together, these results confirm that regardless of the form of RNA Pol. II studied, RNA Pol. II remained associated with the SRC promoter regions and decreased at the 3'UTR in response to TSA treatment. These findings further confirm that TSA treatment may be interrupting transcription at a point after the binding of RNA Pol. II to the promoter regions, thus diminishing the ability of RNA Pol. II to reach the 3' UTR of the SRC gene.

4.2.8. Discussion

4.2.8.1. Histone Acetylation is Not a Simple Predictor of Gene Activity

The transcriptional activation and/or repression -mediated by HDIs can result in apoptosis and cell cycle arrest in numerous neoplasms (Dokmanovic and Marks, 2005). HDI -mediated apoptosis can be achieved through the transcriptional upregulation or downregulation of key factors involved in either the caspase--dependent apoptosis pathway and/or the death receptor apoptosis pathway (Johnstone, 2002). Similarly, HDI induced cell cycle arrest has been suggested to occur through the transcriptional upregulation of the cyclin -dependent kinase $p21^{WAF1}$ combined with the transcriptional downregulation of Cyclin A and D1 (Sandor, *et al*., 2000). While HDIs mediate their effects in a variety of manners, the mechanism responsible for HDI -mediated transcriptional activation and/or repression remains unknown (Chen, *et al*., 2005; Li, *et al*., 2006).

Classically, it has been suggested that HDIs act at the chromatin level to modulate transcriptional activation. This hypothesis was established primarily based on the functions attributed to HDACs. HDACs act, in part, to relieve the acetylation of histone tail lysine residues thereby restoring a positive charge to the lysine residues contained within the histone tail. This increase in positive charge results in increased DNA-histone tail or histone tail-histone interactions, which lead to the condensation of the chromatin. The "closed" conformation marked by condensed chromatin prevents the interactions of gene specific activators and general transcription factors with their cognate DNA sequences thus preventing transcriptional activation (Santos-Rosa and Caldas, 2005). As a result, HDIs are frequently associated with transcriptional activation due to their ability to inhibit HDACs and presumably the repressive activities

of HDACs. In agreement with a role for HDI -mediated acetylation in transcriptional activation, numerous groups have reported increases in histone H3/H4 acetylation at the promoter regions of genes transcriptionally activated by HDIs, most notably at the $p21^{WAF1}$ promoter (Richon, *et al*., 2000; Zhao, *et al*., 2006; Pan, *et al*., 2007). The above hypothesis addresses the mechanism of HDI -mediated transcriptional deregulation at genes whose expression is enhanced by HDIs but fails to identify what is occurring at the promoter regions of genes repressed by HDIs.

Previous studies attempting to identify the mechanisms behind HDI -mediated repression of SRC activity were unsuccessful. Moreover, discrete HDI response elements could not be identified at either promoter region and protein neosynthesis was not required to mediate SRC repression (Kostynuik, *et al*., 2002; Dehm, *et al*., 2004). As a result of these findings, a comparison of the changes of histone acetylation at the promoter regions of two genes differentially affected by HDIs was undertaken. The goal was to identify if there were indeed opposing changes in histone acetylation at the two genes in response to HDIs in order to determine if differential promoter regulation could be explained by differential chromatin modifications.

Surprisingly, it was discovered that regardless of the effect -mediated by HDIs, acetylation of both histones H3 and H4 increased at both SRC promoters and the $p21^{WAF1}$ promoter loci in response to TSA. Although increased histone acetylation was expected at the $p21^{WAF1}$ promoter locus, according to the HDI-chromatin model, acetylation changes at the SRC promoters should have demonstrated an opposite affect. These results suggest that histone acetylation is not an accurate predictor of transcriptional activation as the SRC promoter regions should have demonstrated decreased or unchanged histone acetylation upon HDI treatment. In HepG2 cells, TSA induced increased acetylation at the SRC promoter regions as rapidly as 15 minutes post treatment. The short amount of time elapsed between treatment and an observable change in acetylation is significant as it suggests that effects -mediated by HDIs at the SRC and $p21^{WAF1}$ promoter regions are immediate and likely occurring directly at or within close proximity to the promoter regions.

Further analysis into the discrete residues affected by HDIs resulted in several interesting observations. Specifically, while all three promoters demonstrate similar

overall trends in HDI induced histone acetylation changes in HT29 cells, there were two notable exceptions. The SRC1A and SRC1α promoters are both repressed by HDIs; however, upon treatment with TSA, acetylation at histone H4K12 increased slightly at the SRC1A promoter but decreased at the SRC1α promoter. Moreover, the marginal acetylation of histone H4K12 at the $p21^{WAF1}$ promoter also decreased. These results suggest that the acetylation status of this residue was more similar at the SRC1α and $p21^{WAF1}$ promoter regions, which are differentially affected by HDIs, than at the two SRC promoter regions, which are both repressed by HDIs. Similarly, the same phenomenon was observed with histone H4K16 acetylation; H4K16 acetylation decreased at the SRC1A promoter and increased at the SRC1α and $p21^{WAF1}$ promoter regions. Again, these results suggest greater similarities in histone acetylation patterns between the differentially affected SRC1α and $p21^{WAF1}$ promoter regions. The observation that H4K16 acetylation decreases at the SRC1A promoter is particularly interesting within the context of HDI -mediated transcriptional repression. H4K16 acetylation is reported to prevent the condensation of chromatin by the remodelling enzyme ISWF (Shia, *et al*., 2006). The decrease in H4K16 acetylation at the SRC1A promoter is therefore significant as it provides a possible mechanism by which SRC1A transcription could be repressed through chromatin compaction. However, the increase in H4K16 acetylation at the SRC1α promoter upon treatment suggests that HDI -mediated repression by this mechanism is unlikely. Furthermore, in HepG2 cells H4K16 acetylation increases at both the SRC1A and SRC1α promoter regions. Interestingly, H4K8 acetylation gradually decreased at all three promoter regions studied in HT29 cells. Acetylation of H4K8 has been reported to be required for the recruitment of the chromatin-remodelling enzyme SWI/SNF to contribute to transcriptional initiation at the INF-β locus *in vitro* (Agalioti, *et al*., 2002). Despite compelling studies highlighting unique roles for the acetylation of histone H4 lysine residues in transcriptional activation, a recent study performed in budding yeast suggested that the effects of histone H4K5, K8, and K12 acetylation are cumulative and do not have discreet roles in transcriptional regulation. Conversely, histone H4K16 acetylation was suggested to be required for the transcriptional activation of many genes (Dion, *et al*., 2005), The disparities reported in the consequences -mediated by the

acetylation of histone H4 residues, and the results presented herein, suggest that while acetylation status is changeable, a particular effect -mediated by a change in acetylation is not guaranteed.

The only notable difference in HDI -mediated acetylation changes at the SRC1A and SRC1α promoter regions in HepG2 cells was observed at histone H3K9. H3K9 acetylation increases marginally at the SRC1α promoter but immediately decreases at the SRC1A promoter. This finding is particularly interesting considering that H3K9 acetylation is frequently associated with transcriptionally active genomic regions. In particular, acetylation of both H3K9 and H3K14 mediates the binding of TAF1 via TAF1's double bromodomains and therefore contributes to PIC assembly *in vitro* (Agalioti, *et al*., 2002). The gradual increase of H3K9 and H3K14 acetylation at all three promoter loci upon TSA treatment in HT29 cells suggests that while it is possible that H3K9 and H3K14 acetylation mediates TAF1 binding, the acetylation of these residues does not guarantee subsequent transcriptional activation. Furthermore, in HepG2 cells, H3K14 acetylation increases at both SRC promoters in response to TSA whereas H3K9 acetylation changes are different between the two promoter regions studied. This suggests TAF1 binding is not occurring via these two histone residues in this cell line. Aside from participating in TAF1 binding, however, H3K9 acetylation is mutually exclusive to the methylation of H3K9, which is commonly found in transcriptionally repressed or heterochromatin regions (Eskeland, *et al*., 2007; Wissmann, *et al*., 2007). However, H3K9 trimethylation could not be detected at any of the three promoter regions studied thus suggesting that the chromatin was not condensing into heterochromatin in response to TSA.

Similar to that observed in HT29 cells, the decreased acetylation of H3K9 at the SRC1A promoter did not correlate with the H3K9 acetylation change at the SRC1α promoter in HepG2 cells. This observation, combined with differences in histone H4 acetylation at both SRC promoters in HT29 cells, could suggest a differential mechanism for HDI -mediated repression between the two promoter regions. However, this is unlikely considering the overall histone acetylation status at all three promoter regions studied alters in a similar fashion regardless of HDI -mediated transcriptional events. Therefore, taken together, these studies into the post-translational modifications

of histone residues in response to HDI treatment have shown that changes in histone acetylation are not accurate indicators of transcriptional activation or repression following HDI treatment. Indeed, residue specific alterations in histone acetylation at differentially affected promoter regions did not correlate with transcriptional activation or repression -mediated by HDIs. As a result, the changes in histone acetylation at the SRC promoter regions may simply be an artifact of HDI treatment. Conversely, the increased acetylation may be indicative of the recruitment of co-activators to the SRC promoter regions following HDI treatment. However, in this scenario, HDI -mediated SRC repression would negate the effect of the recruited co-activators by repressing SRC expression at a later stage in transcription.

4.2.8.2. Transcription Factor Binding and RNA Polymerase II Binding to SRC Promoters is Unaffected by TSA Treatment

As HDI -mediated global chromatin deacetylation was not observed at either SRC promoter region, the role of gene specific regulators of transcriptional repression were addressed. Previous studies into HDI-mediated transcriptional activation of $p21^{WAF1}$ suggested a role for the Sp family of factors in HDI induced activation (Xiao, *et al.*, 1999; Huang, *et al*., 2000; Xiao, *et al.*, 2000; Sun, *et al*., 2002). Furthermore, other groups have identified HDI response elements that require Sp factor binding sites for HDI-mediated transcriptional deregulation (Choi, *et al*., 2002; Steiner, *et al*., 2004; Yokota, *et al*., 2004; Huang, *et al*., 2005). These studies all suggest a major role for Sp factor binding in mediating HDI specific effects. However, other studies have suggested HDI -mediated transcriptional repression is the result of abrogated transcriptional initiation through the impeded binding of basal transcription factors such as TBP and RNA Pol. II (Rascle, *et al*., 2003). Similarly, HDI treatment inhibits selected interferon beta (IFNbeta)-stimulated immediate early genes by preventing RNA Polymerase II from binding to promoter regions (Sakamoto, *et al*., 2004). Taken together, these studies suggest that HDI -mediated activation or repression may be mediated through the deregulation of transcriptional initiation. Deregulation could be achieved through either the abrogation, or enhancement of initiation-mediated by sequence specific

transcriptional regulators such as the Sp family, or through the loss of RNA Pol. II binding.

Previous work failed to identify an HDI responsive element at either the SRC1A or SRC1α promoter regions, including at the Sp factor binding sites. However, this same study identified a potential role for general promoter architecture, including both proximal promoter elements and core promoter elements, in HDI-mediated repression (Dehm, *et al*. 2004). In this current study, Sp1, Sp3 and RNA Pol. II occupancy of both the SRC promoters was unchanged in response to TSA treatment. These findings have several interesting implications for HDI -mediated SRC repression.

Despite the well-established involvement of the Sp family of transcription factors in HDI -mediated transcriptional activation, these factors are not involved in HDI -mediated SRC repression. Evidence for this conclusion is drawn from several sources. Firstly, previous studies did not identify an HDI response element at the SRC1A promoter. If HDIs were mediating repression of SRC exclusively through the binding or activity of these factors, mutating the binding sites would diminish the effects of HDI -mediated repression. However, this was not the case (Dehm, *et al*., 2004). Secondly, the Sp family of factors could not be responsible for HDI-mediated repression of the SRC1α promoter, as this family of transcription factors does not activate the SRC1α promoter. Finally, the binding of distal promoter elements by factors such as Sp1 are reported to enhance or disrupt PIC assembly prior to RNA Pol. II binding (Ryu, *et al*., 1999). The finding that RNA Pol. II occupancy at the SRC1A and SRC1α promoter regions was not abrogated by HDI treatment suggests that the majority of the PIC assembles despite TSA treatment. Therefore, maintained RNA Pol. II promoter occupancy suggests that the effects imposed by Sp factor binding, whether positive or negative, have already occurred and were not detrimental to the PIC assembly.

These results are unlike those observed for HDI -mediated repression of both cytokine and interferon induced gene expression (Rascle, *et al*., 2003; Sakamoto, *et al*., 2004). Both cytokine and interferon targeted genes are activated as a result of transcriptional induction. Therefore, RNA Pol. II occupancy at affected promoter regions can be followed prior to induction, when RNA Pol. II should not be present, all

the way through induction and transcriptional activation, when RNA Pol. II should be present. Furthermore, these studies have suggested that the abrogated binding of transcriptional activators STAT5 or interferon regulatory factor 9 (IFR9) are responsible for impeded RNA Pol. II binding to these promoter regions. As evidenced by Sp1/Sp3 ChIP results, however, the binding of transcriptional activators to the SRC1A promoter was not abrogated upon HDI treatment.

Continued RNA Pol. II binding at both SRC promoter regions, despite HDI treatment, suggests that HDIs are affecting either transcriptional initiation or elongation. The presence of RNA Pol. II at the promoter region does not, however, identify if TFIIE and/or TFIIH is(are) binding to the partially assembled PIC to mediate transcription initiation. These studies have, however, shown that RNA Pol. II occupation at the SRC promoter regions was not abolished upon treatment with TSA, which suggests that HDIs affect SRC expression downstream of RNA Pol. II binding.

4.2.8.3. Reduction in RNA Polymerase II Occupancy at Regions 3'of the SRC Promoters in Response to TSA Treatment

Regions downstream of the SRC1A promoter were analysed for RNA Pol. II occupancy. Overall, these results suggest that RNA Pol. II was sequestered at the SRC promoter regions upon TSA treatment as a definite decrease in RNA Pol. II occupancy at the 3' UTR was detected as soon as 30 minutes post-treatment in both HepG2 and HT29 cells. Furthermore, the amount of time required to observe a change in RNA Pol. II occupancy at the SRC 3'UTR was in agreement with the amount of time elapsed before TSA promoter specific effects would be observed at the distal ends of the gene (Neugebauer, 2002; Mason and Struhl, 2005). However, at several regions between the promoter and 3'UTR there were unexpected disparities in RNA Pol. II enrichment at almost adjacent amplicons.

In discussing these discrepancies it is important to note that although HDI - mediated effects are being measured, the differences in RNA Pol. II occupancy at adjacent regions is apparent in untreated cells. This suggests that treatment with HDIs is unrelated to the observed differences. For example, RNA Pol. II occupancy is almost undetectable throughout the time course at the amplicon between exon 1A and 1B.

Whereas, downstream at the amplicon between exon 1B and 1C, RNA Pol. II occupancy was detected at the 0 time point in both cell lines. A similar phenomenon was observed at adjacent amplicons between exon 4 and exon 5. It would be expected that if RNA Pol. II could not be detected upstream at a particular region, that RNA Pol. II would not be detected at an amplicon downstream. This is clearly not the case. The most obvious reason for the observed disparities may reside in differences in PCR efficiency between amplicons. The region between exon 1A and 1B is very GC rich and, as a result, amplification of this region is much more difficult. Moreover, while the adjacent amplicons between exon 4 and exon 5 demonstrate similar decreases in RNA Pol. II occupancy upon HDI treatment PCR products obtained from the second amplicon were clearer. Aside from experimental difficulties, the duration of RNA Pol. II occupancy was not consistent along a gene. An i*n vitro* study into RNA Pol. II pause sites within the c- and N-myc gene suggested that a pause in RNA Pol. II transcription frequently occurs in three distinct gene regions. In particular these regions included regions directly downstream of T-rich regions which yield U-rich mRNA, regions that were U- or C-rich and finally, sites following RNA hairpins (Keene, *et al*., 1999). If one or more of the SRC amplicons analysed encompassed an RNA Pol. II pause site, RNA Pol. II ChIP results would be enriched at this particular region as RNA Pol. II would be present at this particular site for a longer period of time. Moreover, assuming that transcription initiation by RNA Pol. II was consistently occurring prior to treatment, and by logical extension, that productive elongation was occurring, a relative backlog of RNA Pol. II moieties would be present at regions upstream from the site of transcriptional pausing. This scenario may explain why RNA Pol. II occupancy at particular regions along the SRC gene may be better represented by ChIP analysis than RNA Pol. II occupancy at other regions.

4.2.8.4. No Change in RNA Polymerase II Phosphorylation at SRC Promoters upon TSA Treatment

Experiments measuring RNA Pol. II occupancy at the SRC promoter regions and more distal regions of the gene show transcriptional initiation or elongation by RNA Pol. II was impeded upon HDI treatment. Moreover, studies utilizing antibodies specific for the phosphorylated forms of RNA Pol. II demonstrated that transcriptional

initiation was likely occurring, which suggests that productive transcriptional elongation was likely abrogated by HDIs.

The logic used in the derivation of these conclusions stems from the observation that the occupancy of RNA Pol. II CTD phosphorylated at serine 5 (S5) is maintained at the SRC promoter regions despite treatment with TSA. RNA Pol. II S5 phosphorylation is -mediated exclusively through the kinase activity of TFIIH (Svejstrup, 2003; Thomas and Chiang, 2006). The phosphorylation of S5 suggests that TFIIH and TFIIE are present within the PIC as TFIIE is responsible for stimulating the ATPase, CTD kinase and helicase activities of TFIIH to initiate RNA Pol. II -mediated transcription (Thomas and Chiang, 2006). Moreover, the phosphorylation of S5 within the CTD of RNA Pol. II is frequently associated with and required for initiated transcription (Komarnitski, *et al*., 2000; Svejstrup, 2003; Meinhart, *et al*., 2005). Therefore, the lack of change in the occupancy of S5 phosphorylated RNA Pol. II, upon HDI treatment, suggests that TSA was not repressing SRC expression via inhibition of transcriptional initiation. If transcriptional initiation was abrogated by HDI treatment, a decrease in RNA Pol. II S5 phosphorylation would be observed as soon as 15 minutes post-treatment. This was not the case.

As expected, the detection of the S2 phosphorylated form of RNA Pol. II at either SRC promoter regions was difficult. S2 phosphorylated RNA Pol. II is more frequently observed at regions downstream of the promoter as S2 phosphorylation occurs after the addition of the methylated guanosine cap to the nascent mRNA (Saunders, *et al*., 2006). The requirement of such co-transcriptional processes in the phosphorylation of S2 RNA Pol. II, have led to the acceptance that S2 phosphorylated RNA Pol. II is indicative and required for transcriptional elongation (Komarnitski, *et al*., 2000; Svejstrup, 2003; Meinhart, *et al*., 2005). However, by performing quantitative PCR it was demonstrated that S2 phosphorylated RNA Pol. II was present at SRC promoter proximal regions, although at a very low abundance. Upon treatment with TSA the occupancy of this form of RNA Pol. II did not change. The lack of change in distribution of this form of RNA Pol. II following TSA treatment would generally indicate that elongation was not impeded by TSA. However, the use of changes in S2 phosphorylated RNA Pol. II occupancy at promoter regions as an indicator of deviated

transcriptional elongation was not ideal, as the levels of S2 phosphorylated Pol. II were already very low in these regions. Therefore, subsequent decreases in phosphorylation were difficult to gauge. Indeed, work with the inducible MAP kinase phosphatase-1 (MKP-1) gene has suggested that the induced phosphorylation of S2 RNA Pol. II was best observed at regions far downstream of the promoter (Fujita, *et al*., 2007). As expected, the S2 phosphorylated form of RNA Pol. II was abundant at the 3'UTR of SRC prior to TSA treatment. However, in agreement with previous RNA Pol. II occupancy ChIP results, the distribution pattern of the S5 phosphorylated form of RNA Pol. II and S2 phosphorylated from of RNA Pol. II decreased at the distal 3' regions of the gene upon TSA treatment. These results suggest a model whereby transcriptional elongation, not initiation, is attenuated by these drugs.

In agreement with a role for impeded transcriptional elongation in HDI -mediated SRC repression, previous transient transfection experiments performed with SRC reporter constructs were susceptible to HDI -mediated repression (Kostyniuk, *et al*., 2002; Dehm, *et al*., 2004). If HDIs were repressing SRC transcriptional activity via chromatin remodelling, the transiently transfected reporter constructs would likely be immune to the effects -mediated by HDIs as these would target chromatin packaged endogenous SRC. In addition, these SRC reporter constructs are composed of a relatively small region of the SRC promoter DNA. Therefore the effect imposed by HDIs could be -mediated at only this small region of the SRC promoter locus. Interestingly, the segment of the SRC locus contained within these reporter constructs encompasses the region upstream of the transcriptional start site, which is required for the binding of transcription factors, the Inr element, as well as the region downstream of the transcriptional start site. As these experiments have already eliminated the abrogated binding of transcription factors and/or RNA Pol. II as a mechanism for SRC repression by HDI treatment, only one region and the processes associated with this region remains as a target for HDI -mediated SRC repression. Therefore by process of elimination, the only remaining step that could be deregulated to result in abrogated SRC promoter activity following TSA treatment is elongation.

HDIs could be attenuating transcriptional elongation at any of several critical stages in elongation. A key rate-limiting step in the transition from transcriptional

initiation to the formation of the early elongation complex revolves around the stabilization of the 4-5 nucleotide nascent transcript by TFIIB (Chen and Hampsey, 2004; Saunders, *et al.*, 2006). Conversely, prolonged TFIIB association with the very early elongation complex can impede the "bubble" collapse necessary to produce energy required for productive elongation thus preventing promoter escape. Indeed, *in vitro* assays have suggested that "bubble" collapse and elongation between nucleotide +7 and +9 is attenuated by the presence of TFIIB (Pal, *et al*., 2005). Clearly, the association of TFIIB with the initiation and elongation complexes must be finely regulated to ensure proper associations at appropriate phases of transcription. Interestingly, the autoacetylation of TFIIB, which enhances the stability of TFIIB/TFIIF interactions, potentially contributes to transcriptional activation (Choi, *et al*., 2003). HDI treatment could prevent the deacetylation of TFIIB, thereby prolonging an interaction with TFIIF, which could eventually prevent promoter escape by the elongation complex. While possible, this scenario is unlikely as it has been suggested that the deacetylation of TFIIB occurs without the aid of a deacetylase (Choi, *et al*., 2003).

A more likely mechanism for HDI -mediated repression of SRC transcriptional elongation involves deregulation of promoter-proximal pausing (Figure 4.21.). DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) bind to the early elongation complex (EEC) thus pausing the complex to facilitate the recruitment of the capping enzyme (CE) to the S5 phosphorylated CTD of RNA Pol. II (Sims III, *et al.*, 2004). Elongation continues as a result of the association of positive transcription-elongation factor-b (P-TEFb) with the stalled elongation complex, which mediates the phosphorylation of S2 residue within the CTD as well as DSIF and NELF (Saunders, *et al*., 2006). The phosphorylation of NELF causes its disassociation from the paused complex and the phosphorylation of DSIF converts DSIF into a positive elongation factor (Peterlin and Price, 2006). Concomitantly, the small CTD phosphatase (SCP) dephosphorylates the S5 residue within the CTD. These alterations in the elongator complex recruit TFIIS to contribute to the re-initiation of elongation (Sims III, *et al*., 2004). The elegant interplay of enzymatic functions culminating in the formation of the mature elongation complex could be easily undone through the deregulation of one key

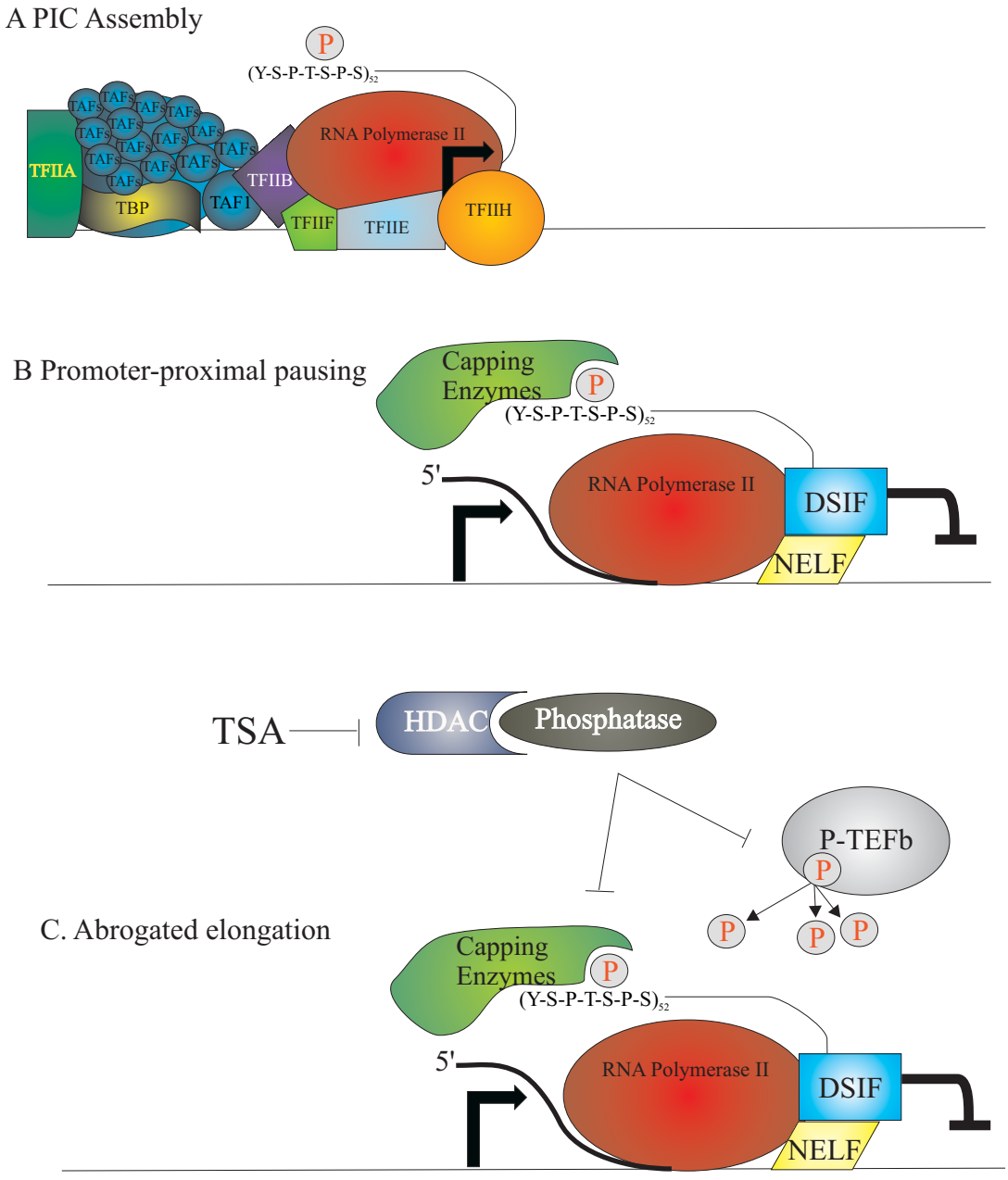


Figure 4.21. Model of TSA mediated abrogation of transcriptional elongation. A. Pre-initiation complex (PIC) fully assembled for transcription initiation. B. Promoter-proximal pausing of RNA Pol. II by DSIF and NELF to accommodate the entry of the capping enzymes to the S5 phosphorylated CTD. C. TSA inhibits HDAC activity and disrupts phosphatase/HDAC complex formation. Phosphatase dephosphorylates P-TEFb to inactivate P-TEFb kinase domain to prevent subsequent P-TEFb kinase activity thus preventing RNA Pol. II release form promoter-proximal pausing. Conversely, phosphatase aberrantly dephosphorylates another key elongation dependent factor.

enzyme. For example, the association of P-TEFb with the paused elongation complex and subsequent kinase functions of P-TEFb strongly depends on the phosphorylation of the CDK9 subunit of P-TEFb. If the phosphorylation of P-TEFb is compromised, full enzymatic activity is lost and the early elongation complex is not released from the pause site (Peterlin and Price, 2006). Significantly, it has recently been demonstrated that HDI treatment disrupts protein phosphatase 1 (PP1)/HDAC complexes resulting in the dephosphorylation of effectors (Chen, *et al*., 2005; Alao, *et al*., 2006). Interestingly, the CDK9 subunit of P-TEFb is a substrate for PP1 -mediated dephosphorylation (Ammosova, *et al*., 2005). It is therefore not difficult to imagine that HDI treatment could release PP1 from HDAC complexes, to dephosphorylate P-TEFb thus preventing re-initiation of the stalled elongation complex and ultimately, abrogate SRC expression. Similarly, PP1 also dephosphorylates the S2 CTD residue of RNA Pol. II *in vitro*, providing another potential target for HDI -mediated repression via curtailed elongation (Washington, *et al*., 2002). The premature dephosphorylation of any of the components of the stalled elongation complex could result in aberrant expression. Interestingly, other studies have suggested a role for kinases in HDI -mediated transcriptional activation of $p21^{WAF1}$, NF-κβ and gelsolin (Han, *et al*., 2001; Kim, *et al*., 2006; Eun, *et al*., 2007). The observations that kinases are involved in mediating transcriptional activation by HDIs further supports an argument for a role for phosphatases in mediating HDI repression. Moreover, it is important to consider that the interaction between elongation factors and phosphatases is only one example of a potential mechanism by which HDIs could repress expression through reduced transcriptional elongation. There are likely many such interactions that could be deregulated or interrupted by HDI treatment.

4.2.8.5. Scope and Significance

HDIs are a powerful and exciting class of chemotherapeutic agents credited with inducing apoptosis, cell cycle arrest and terminal differentiation in cancer cells. The mechanisms behind these effects are only beginning to emerge, however, the mechanism behind the transcriptional activation or repression by HDIs remains unknown. Classically, HDI -mediated histone modifications, such as acetylation, were credited with altering chromatin condensation to enhance transcriptional activation.

However, current studies suggest that HDI -mediated histone modifications are not necessarily indicative of the subsequent transcriptional activity of a particular gene. Furthermore, studies of inducible genes have suggested a role for abrogated RNA Pol. II binding in mediating transcriptional repression by HDIs, as a result of impeded transcription initiation. As an equal number of genes are repressed by HDIs as activated by these agents, the utility in discovering the mechanism for HDI -mediated repression cannot be emphasized enough. This current work suggests that transcription elongation, not initiation, may be responsible for HDI -mediated curtailed SRC transcriptional expression. Furthermore, as many pertinent genes are repressed by HDIs, such as cyclin A and cyclin D1, these findings could provide powerful clues in deciphering the general mechanism of HDI -mediated transcriptional repression.

4.3. REQUIREMENT OF HDAC ACTIVITY FOR SRC TRANSCRIPTIONAL ACTIVATION

4.3.1. Effect of Class I HDACs on the Promoter Activity of SRC and p21^{WAF1}

Recent studies have suggested that in some cases an HDAC may be required for transcriptional activity (Rascle, *et al*., 2003; Klampfer, *et al*., 2004). This finding correlates well with observations that SRC is repressed by HDIs. Indeed, if an HDAC is required for SRC transcription, and HDIs are inhibiting the essential HDAC, an abrogation of transcriptional activation would be expected. Therefore in an attempt to identify if HDACs are required for the transcriptional activation of SRC, a series of knockdown and overexpression experiments were performed with HDACs.

Class I HDACs are ubiquitously expressed, inhibited by HDIs and frequently associated with transcriptional regulation (Yang and Seto, 2003). In an attempt to identify if any of the class I HDACs were responsible for the TSA-mediated effects on SRC, all of the class I HDACs were individually overexpressed in SW480 and HepG2 cells. These experiments were performed in an effort to identify what effect, if any, these factors have on SRC transcriptional activity. Furthermore, if a class I HDAC is required for SRC promoter activity, the overexpression of each individual class I HDAC should result in increased SRC promoter activity. As described in a previous section, p21^{WAF1} is transcriptionally activated by TSA. Furthermore, HDAC2 has been reported to directly repress p21^{WAF1} promoter activity (Huang, *et al.*, 2005). Therefore as a proof of principle experiment, class I HDACs were co-transfected with the p21 reporter construct into SW480 cells (Fig. 4.22.). While HDAC2 and 8 could repress p21^{WAF1} promoter activation, HDAC3 overexpression had little effect and surprisingly, HDAC1 could modestly activate p21^{WAF1}. These experiments confirmed that the overexpression of a class I HDAC was capable of regulating promoter activity. Co-transfection of SW480 cells with the SRC reporter constructs and each HDAC had differing effects (Fig. 4.23. A.). HDAC1, 2, 3 and 8 all decreased SRC1A promoter activity in SW480 cells, whereas, SRC1α activity remained the same or decreased slightly upon co-transfection with the class I HDACs in this cell line. In contrast, none of the class I HDACs had any effect on SRC activation, from either promoter, in HepG2 cells (Fig. 4.23. B.).

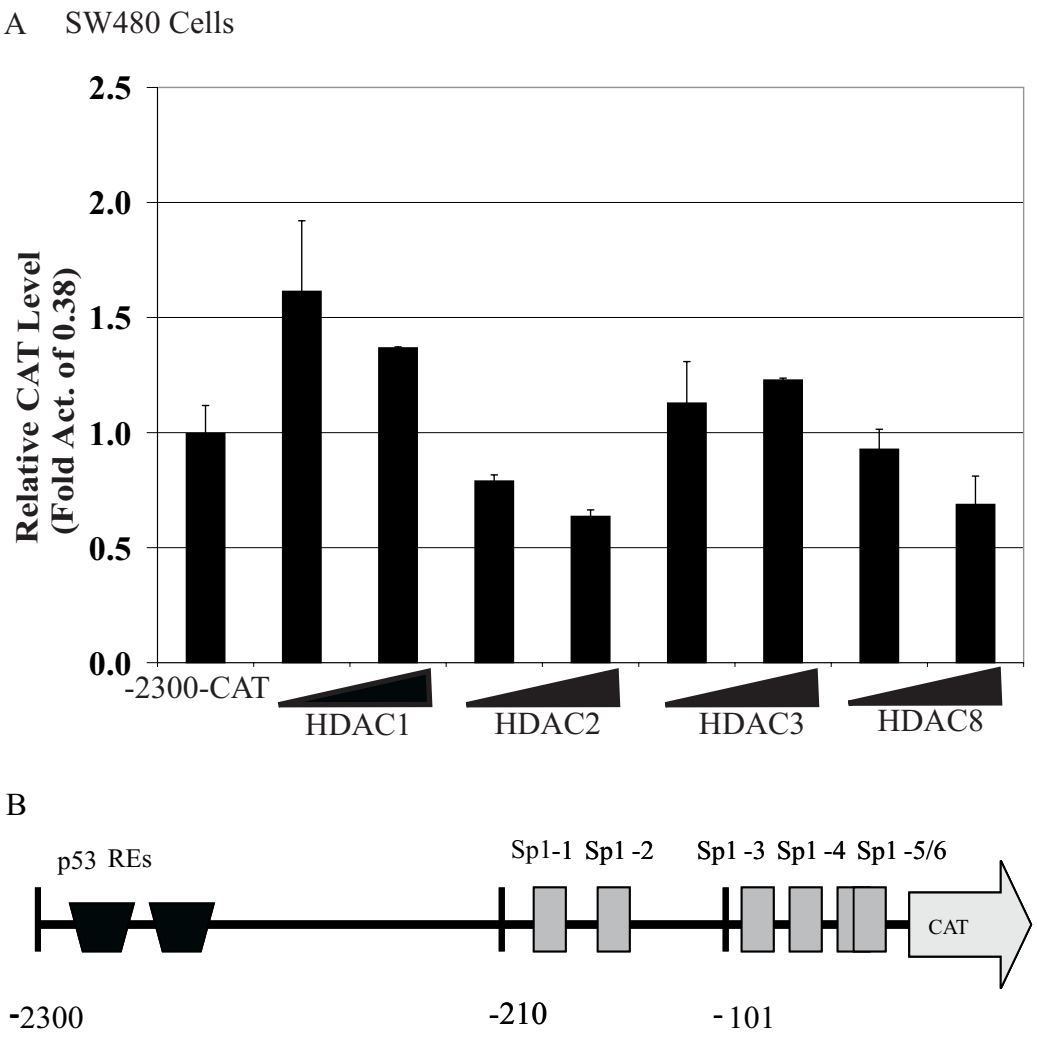


Figure 4.22. Effect of the overexpression of Class I HDACs on the p21^{WAF1} promoter. (A) Class I HDACs (HDAC1, 2, 3 and 8) and p21WAF1-CAT were co-transfected into SW480 cells.(B) The p21WAF1-CAT reporter construct used in A. These results are representative of experiments performed at least three times in duplicate. The standard deviation was calculated from two experiments each performed in duplicate.

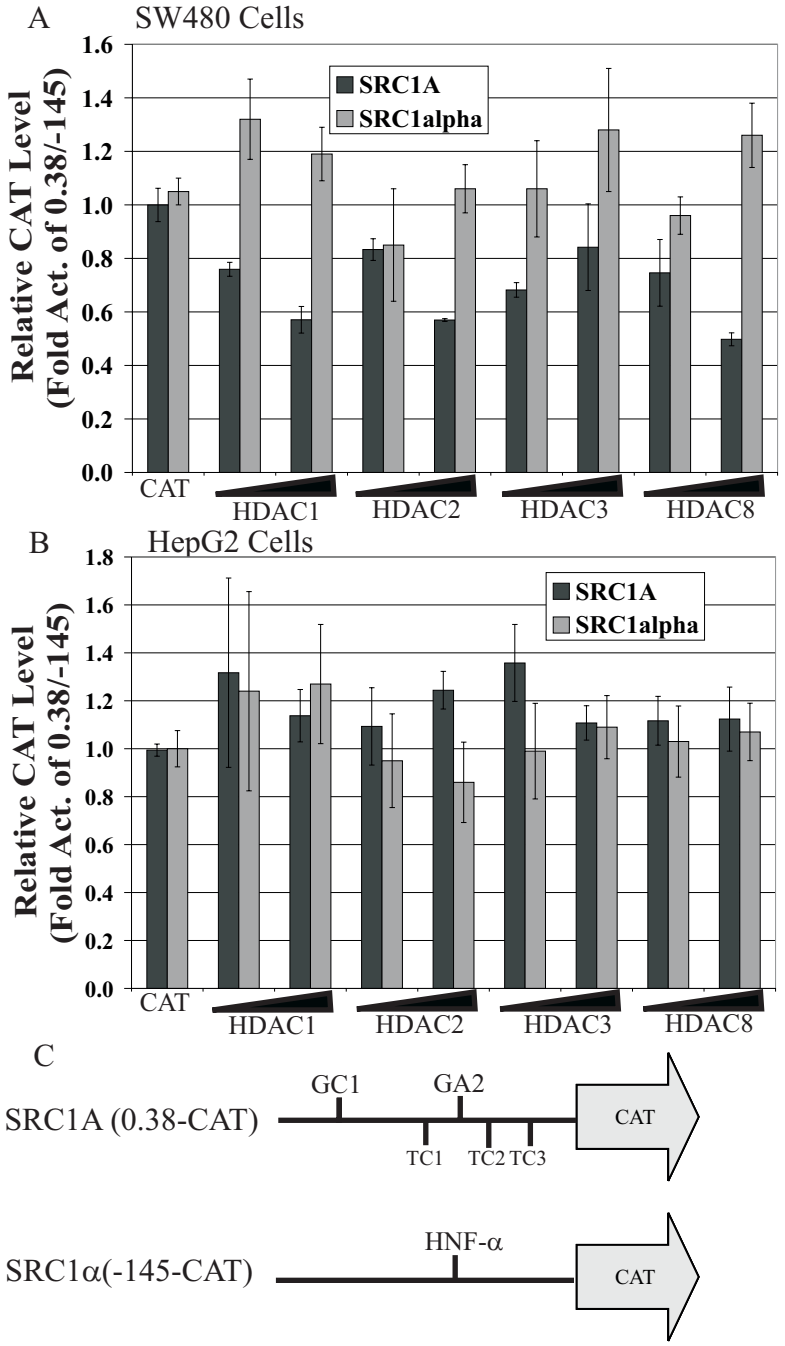


Figure 4.23. Effect of the overexpression of Class I HDACs on the SRC1A and SRC1α promoters. (A) Class I HDACs (HDAC1, 2, 3 and 8) and SRC1A-CAT or SRC1α-CAT were co-transfected into SW480 cells.(B) Class I HDACs (HDAC1, 2, 3 and 8) and SRC1A-CAT or SRC1α-CAT were co-transfected into HepG2 cells. (c) The SRC1A-CAT and SRC1α-CAT reporter constructs used in A and B. These results are representative of experiments performed at least three times in duplicate. The standard deviation was calculated from two experiments each performed in duplicate.

4.3.2. Effect of Class I HDACs Knockdown on SRC Expression Levels

To complement the class I HDAC transfection studies, siRNA -mediated knockdowns of all of the class I HDACs were performed in HepG2 and HT29 cells (Fig. 4.24.). siRNA transfected cells were harvested and total RNA was extracted from the samples. RT-PCR was performed with primers specific for the HDAC being knocked down, SRC and a positive loading control (RPL13A). The knockdown of all four class I HDACs resulted in increased SRC expression in both cell lines studied, although the effect was clearest in HepG2 cells. Notably, an increase in SRC message was most apparent when HDAC8 was knocked down. This observation may be particularly significant as HDAC8 was not abundant prior to knockdown, suggesting that only a slight decrease in HDAC8 may result in dramatic increases in SRC activation.

Taken together, these results suggest that none of the class I HDACs are involved in SRC transcriptional activation. Moreover, it appears that all four of the class I HDACs may have a negative role in SRC regulation.

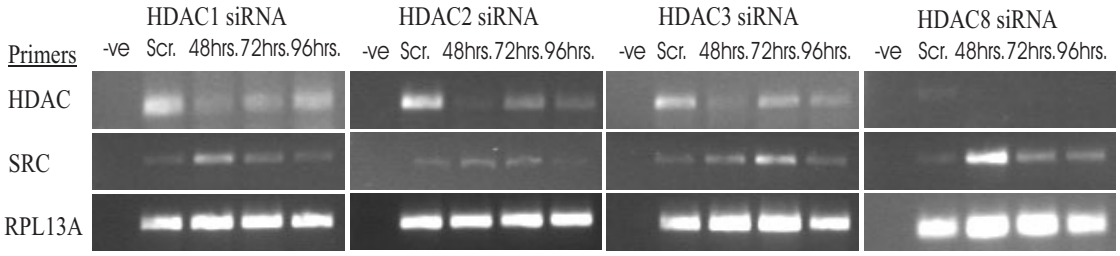
4.3.3. Effect of Class II HDACs Knockdown on SRC Expression

Class II HDACs are also inhibited by HDIs and, though not as well studied, are also reported as having a role in transcriptional regulation (Fischle, *et al*., 2001). To characterize what role class II HDACs exerted on SRC expression, siRNA -mediated knockdowns of HDAC 5, 6, 7 and 10 were performed in HepG2 and HT29 cell lines followed by RT-PCR (Fig. 4. 25.). HDAC4, 5, 6, 7, 9 and 10 are all described as class II HDACs but as preliminary RT-PCR analysis failed to produce a discernable signal for HDAC4 and 9 in the cell lines studied, these two representatives were omitted from further investigation. Interestingly, in HepG2 cells, the knockdowns of all four class II HDACs studied eventually resulted in decreased SRC mRNA. This was not as apparent in HT29 cells, however, although the decrease in SRC mRNA could still be observed if the scrambled and 96 hours time points were directly compared.

Class I HDACs

A

HepG2 cells



B

HT29 cells

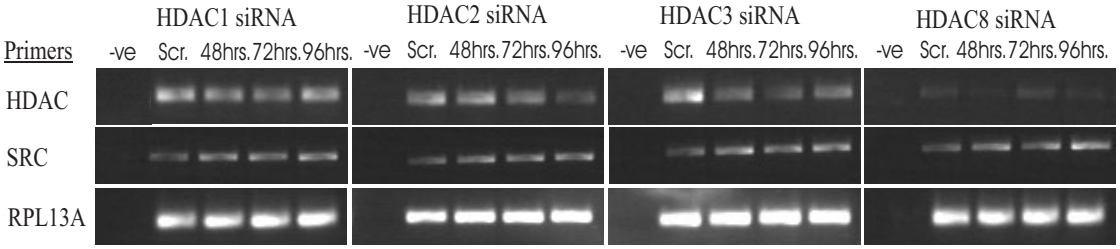
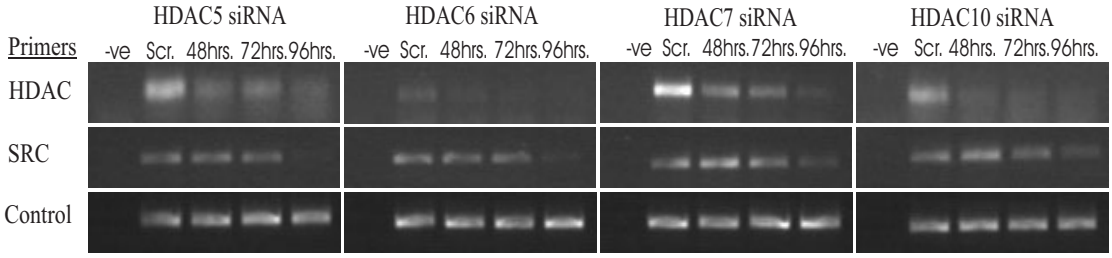


Figure 4.24. Class I HDACs may repress SRC transcriptional activity. HepG2 cells (A) and HT29 cells (B) were transfected with 100 nM of siRNA specific for each class I HDAC (HDAC1, 2, 3 and 8). Transfected cells were harvested at 48, 72 and 96 hours time points, RNA was extracted and RT-PCR was performed with primers specific for the HDAC in question, SRC and RPL13A (positive control). All results were compared to the scrambled (Scr.) siRNA transfected samples. The negative RT-PCR control was distilled water. These are representative of experiments performed at least three times in each cell line.

Class II HDACs

A

HepG2 cells



B

HT29 cells

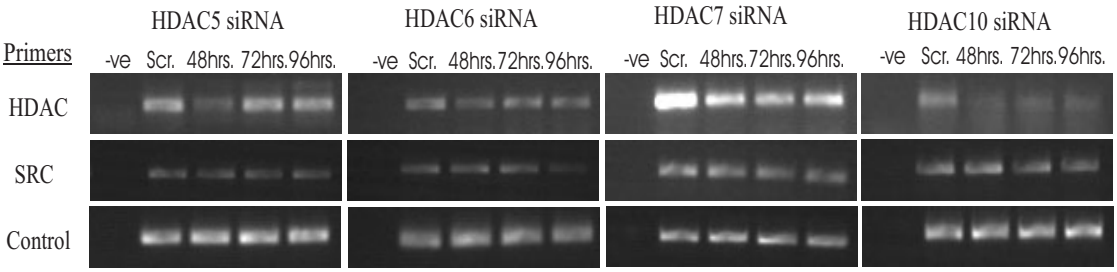


Figure 4.25. Class II HDACs may be neccessary for SRC expression. HepG2 cells (A) and HT29 cells (B) were transfected with 100 nM of siRNA specific for each class II HDAC (HDAC5, 6, 7 and 10) expressed in the above cell lines. Transfected cells were harvested at 48, 72 and 96 hours time points, RNA was extracted and RT-PCR was performed with primers specific for the HDAC in question, SRC and RPL13A (positive control). All results were compared to the scrambled (Scr.) siRNA transfected samples. The negative RT-PCR control was distilled water. These are representative of experiments performed at least three times in each cell line.

4.3.4. Effect of Class II HDAC on SRC Promoter Activity

The siRNA -mediated knockdown of select class II HDACs resulted in decreased SRC mRNA thus suggesting a role for any or all of these HDACs in the positive regulation of SRC. To identify if these class II HDACs were capable of acting directly at the SRC promoter regions to increase transcript level, co-transfection studies similar to the class I HDAC studies were performed in HepG2 and SW480 cells (Fig. 4. 26.). In SW480 cells, HDAC5 and 7 did not change the activity of either promoter. However, in the same cell line, HDAC6 and 10 overexpression resulted in decreased SRC transcriptional activation via both SRC promoters. Conversely, in HepG2 cells, HDAC5, 7 and 10 overexpression all resulted in decreased SRC promoter activity. HDAC6 overexpression caused no change in SRC transcriptional activity at either promoter in either the cell lines studied.

The overexpression results indicate that a class II HDAC may not be required for SRC transcriptional activation. However, the RT-PCR data suggests that the class II HDACs may have an indirect role in maintaining the SRC transcript levels.

4.3.5. Discussion

Several recent studies have described a role for histone deacetylases (HDACs) in transcriptional activation in both human and yeast cells (Vidal, *et al*., 1991; Rascle, *et al*., 2003; Xu, *et al*., 2003; Chang, *et al*., 2004; Kato, *et al.*, 2004; Klampfer, *et al*., 2004; Sakamoto, *et al.*, 2004; Nusinzon and Horvath, 2006; Sharma, *et al*., 2007). For example, in STAT 5 activated expression, HDAC activity was required for the recruitment of the basal transcription factors TBP and RNA Pol. II to promoter regions upon cytokine stimulation. Indeed, treatment with HDIs impeded STAT5 activated transcription in all genes analysed (Rascle, *et al*., 2003). Similarly, STAT5 induced expression of the Id-1 gene required HDAC1 activity to deacetylate transcription factor C/EBPβ (CCAAT/enhancer binding protein-α) to promote activation (Xu, *et al*., 2003). A general role for HDACs in mediating the activation of interferon-stimulated genes has also been established, however, the precise mechanism has yet to be determined. One study suggests that HDAC activity is required for RNA Pol. II binding to the

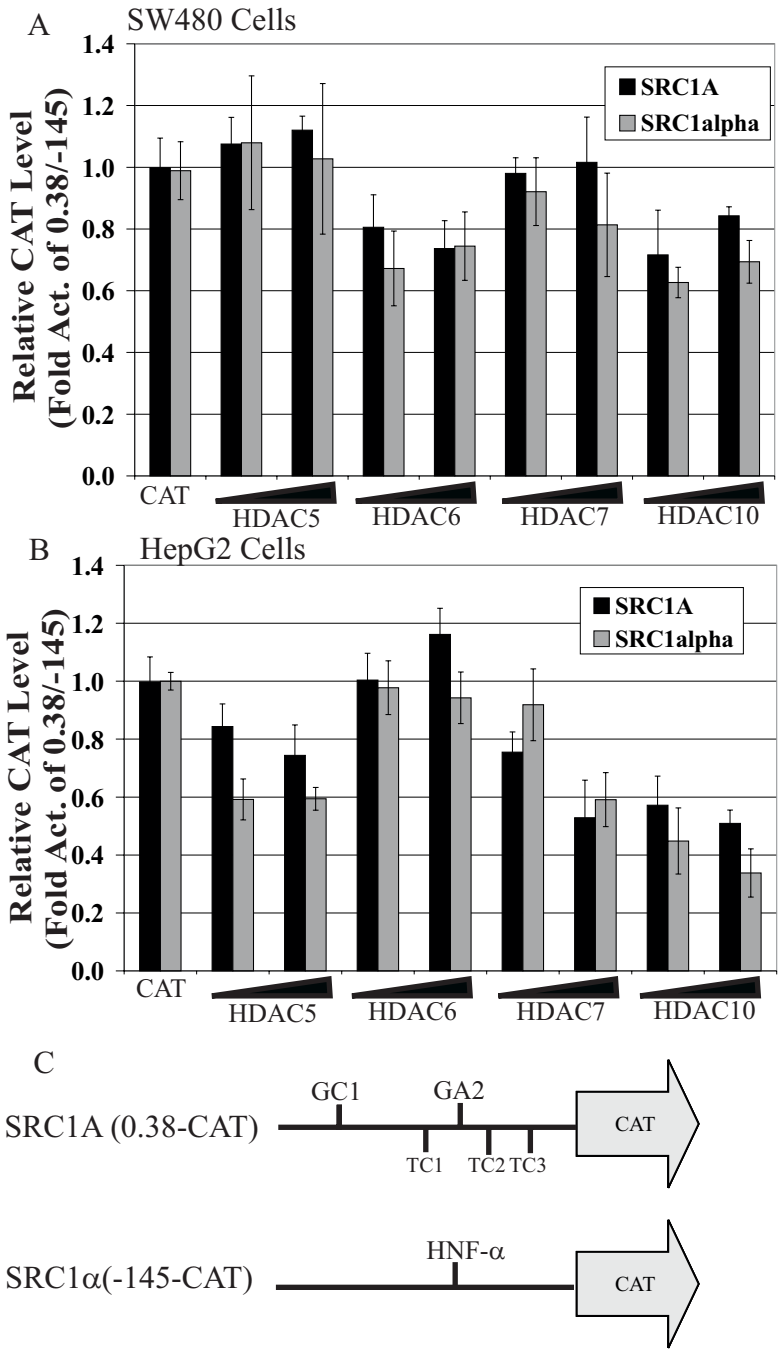


Figure 4.26. Effect of the overexpression of Class II HDACs on the SRC1A and SRC1α promoters. (A) Class II HDACs (HDAC5, 6, 7 and 10) and SRC1A-CAT or SRC1α-CAT were co-transfected into SW480 cells.(B) Class II HDACs (HDAC5, 6, 7 and 10) and SRC1A-CAT or SRC1α-CAT were co-transfected into HepG2 cells. (c) The SRC1A-CAT and SRC1α-CAT reporter constructs used in A and B. These results are representative of experiments performed at least three times in duplicate. The standard deviation was calculated from two experiments each performed in duplicate.

ISG54 promoter to activate transcription (Sakamoto, *et al.*, 2004). In another study, a role for HDAC 6 in the activation of INF-β gene expression has been suggested (Nusinzon and Horvath, 2006). Furthermore, HDIs have also been shown to inhibit INFγ-induced STAT1--dependent transcription through decreased HDAC1, 2 and 3 activity, thereby suggesting a requirement for these HDACs in STAT-1 -dependent transcription (Klampfer, *et al*., 2004). Finally, HDAC 7 has been shown to co-localize with HIF-1α in the nucleus under hypoxic conditions to promote the transcriptional activation of HIF-1α responsive genes (Kato, *et al.*, 2004). These studies conflict with the general paradigm of a repressive role for HDACs in transcriptional activation, as well as provide a potential mechanism for HDI-mediated repression.

The studies described in the previous sections have suggested that HDI -mediated repression of SRC is not achieved though impaired transcription factor binding, histone specific changes in acetylation, or impeded transcription initiation. Furthermore, despite identifying a potential role for attenuated transcriptional elongation in HDI -mediated SRC repression, the actual mechanism(s) behind SRC transcriptional repression by HDIs has not been determined. Though initially counter-intuitive, the requirement of an HDAC for SRC transcriptional activation may explain how HDIs are able to repress SRC mRNA expression. In agreement with this hypothesis, $p21^{WAF1}$ transcriptional activation has been shown to be repressed by HDAC1, 2 and/or 3 (Wilson, *et al*., 2006). As discussed in previous sections, $p21^{WAF1}$ is the classical example of a gene that is transcriptionally activated by HDIs and, as such, a role for HDACs in transcriptional activation provide a potential mechanism for HDI -mediated repression in gene expression.

4.3.5.1. Class I HDACs do not Activate SRC Promoters

When studies examining the overexpression of all four class I HDACs were carried out it was found that none of the class I HDACs were able to activate SRC from either promoter region. Furthermore, that the SRC promoters were affected differently by overexpression of the class I HDACs suggests that a class I HDAC is not required for SRC activation, much less responsible for HDI -mediated repression. Interestingly, in SW480 cells, the SRC1A promoter is repressed by the overexpression of all four class I HDACs. The SRC1A selective decrease in activity could be explained by the Sp

family -mediated regulation of the SRC1A promoter. The abrogated interaction between Sp1 and class I HDACs is a frequent finding at genes that are transcriptionally activated by HDIs, suggesting that HDAC/Sp1 interactions are generally repressive to these genes (Xiao, *et al.*, 1999; Huang, *et al*., 2000; Xiao, *et al.*, 2000; Yokota, *et al*., 2004; Huang, *et al*., 2005). As such, the overexpression of HDACs in SW480 cells may have led to increased associations between Sp1/Sp3 and the class I HDACs at the SRC1A promoter, resulting in transcriptional repression. Previous findings suggesting that Sp1/Sp3 do not contribute to HDI -mediated repression of SRC do not preclude these factors from interactions that mediate SRC1A repression in-dependent of HDIs. In agreement with a role for Sp/HDAC interactions repressing SRC1A activity, the SRC1α promoter, which is not regulated by Sp1/Sp3, is unaffected by the overexpression of class I HDACs in this cell line. Surprisingly, siRNA -mediated knockdown of the class I HDACs resulted in increased SRC message 48 hours post-transfection, which further suggests that class I HDACs are repressive to SRC expression. Although the HDAC knockdown -mediated increase in SRC transcript levels was observed in both cell lines studied, it was easier to observe in HepG2 cells due to the ease with which these cells respond to transfection. If both the level of HDAC knockdown and increase in SRC message are analysed in HT29 cells, proportional changes in message can be observed. These results suggest that while SRC is repressed by HDIs, it is not due to a requirement of class I HDACs for transcriptional activation. In fact, class I HDACs appear to have a negative influence on SRC expression.

4.3.5.2. Class II HDACs do not Activate SRC Promoters but may be Required for the Maintenance of SRC mRNA Levels

HDIs, in particular TSA, also repress class II HDACs (Bolden, *et al*., 2006). To fully examine the potential role of HDACs in SRC transcriptional regulation, class II HDAC activity was also addressed. In contrast to results obtained from class I HDAC knockdown studies, siRNA -mediated knockdown of class II HDACs eventually resulted in a decrease in SRC expression. This was especially apparent when HDAC6 is knocked down in both cell lines analyzed. To identify if class II HDACs directly affected SRC transcriptional activation, overexpression studies were again performed. If class II HDACs were involved in SRC transcriptional activation, an increase in SRC

activity might be expected upon class II overexpression. This was not the case, suggesting that while this class of HDAC may be involved in maintaining SRC expression, as suggested by the decrease in SRC mRNA following class II knockdown, they are not involved in the transcriptional activation of SRC. Conversely, an HDAC may be required for promoter activity, however, the overexpression of an HDAC in transient transfections may be insufficient for defining the role of the particular HDAC in SRC promoter activity. Overexpressing a particular HDAC may be problematic as the HDAC in question may already be present at the SRC promoter regions and therefore the presence of more HDAC protein would be redundant. Furthermore, HDACs often function in complex with other proteins and as such the overexpression of an HDAC alone may also be insufficient to achieve a promoter specific effect. As a result of these differing scenarios, the knockdown studies of the class II HDACs were far more informative about what role these HDACs may mediate in SRC promoter activity.

A role for both class I and II HDACs in mediating gene activation has been observed in several interferon, viral and hypoxia induced genes (Rascle, *et al*., 2003; Xu, *et al*., 2003; Chang, *et al*., 2004; Kato, *et al.*, 2004; Klampfer, *et al*., 2004; Sakamoto, *et al.*, 2004; Nusinzon and Horvath, 2006). The same cannot be applied to SRC transcriptional activation, however, as the studies outlined herein have demonstrated that although HDIs mediate the repression of SRC expression, the overexpression of either class I or II HDACs did not activate SRC expression from either the SRC1A or SRC1α promoters. The most plausible explanation for this phenomenon involves the nature of the genes activated by HDACs. Indeed, without exception, all of the genes activated by HDACs are inducible by interferon, viral activity or hypoxic conditions, SRC is not.

Several important conclusions can be drawn from these studies. Firstly, SRC expression appears to be negatively impacted by class I HDACs, as evidenced by increased SRC message upon class I knockdown. Secondly, class II HDACs, in particular HDAC6, have an essential role in the maintenance of SRC expression. Finally, SRC promoter activity is not activated in response to class II HDAC overexpression, thus suggesting that the role of class II HDACs in SRC expression is

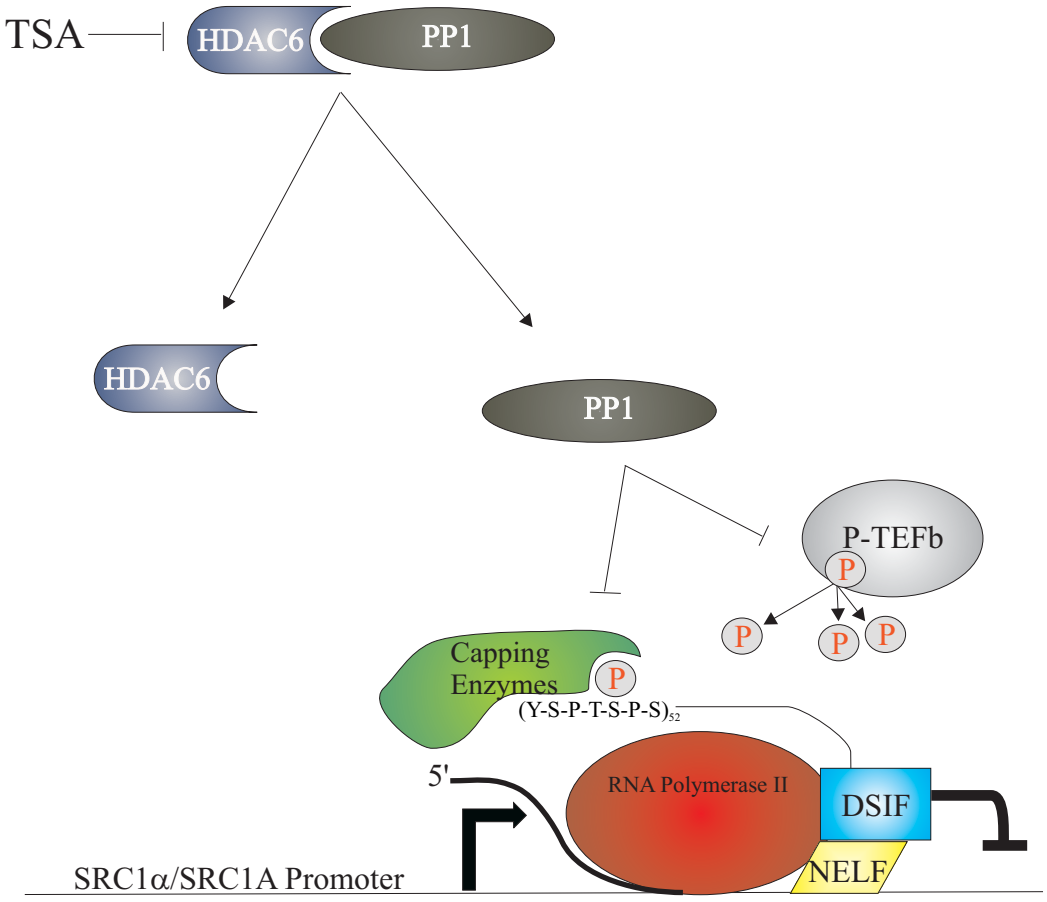


Figure 4.27. Model for HDI mediated SRC repression. Treatment of cells with histone deacetylase inhibitor TSA interrupts the HDAC6/PP1 complex. PP1 subsequently dephosphorylates components of the elongation complex to prevent the re-initiation of elongation. Potential PP1 targets include, P-TEFb, DSIF, NELF and/or S2 CTD RNA Pol. II. Transcription is stalled at SRC promoter-proximal regions therefore decreasing expression in a TSA dependent manner.

not direct. The most obvious implication of these findings is that HDIs may be inhibiting essential class II HDAC processes to attenuate SRC expression. These findings fit well with a model for HDI -mediated repression of SRC whereby HDIs abrogate HDAC/phosphatase complexes to result in the aberrant dephosphorylation of factors critical to transcriptional elongation (Fig. 4.27.). Though a decrease in SRC transcript is not observed until 96 hours post Class II HDAC knockdown, the effect on SRC mRNA may not be observed until the existing class II HDAC protein levels decrease and the implications of class II HDAC loss on SRC expression can be realised. It is also significant that HDAC6 knockdown was most effective in decreasing SRC message in both cell lines tested as HDAC6 has been reported to exist in complex with PP1. Moreover, the HDAC6/PP1 complex is reported to be disrupted by HDI treatment, further supporting a role for both HDAC6 and PP1 in SRC repression via abrogated transcriptional elongation (Chen, *et al*., 2005; Alao, *et al*., 2006).

4.3.5.3. Scope and Significance

The full extent of effects -mediated by HDACs are only now being realised. Originally only considered within the context of chromatin remodelling -mediated by histone specific modifications, HDACs are now known to be involved in transcriptional activation and repression, as well as in a variety of signalling cascades independent of histone modifications. This new found wealth of knowledge concerning HDACs is mostly the result of studies involving the use of the powerful chemotherapeutic known as histone deacetylase inhibitors. Significantly, the relationship between SRC expression and class II HDACs may be essential in delineating the mechanism of repression -mediated by HDIs. As it has been suggested that the expression of up to 22% of all genes may be affected by HDIs, elucidating the mechanism(s) behind the transcriptional activation or repression -mediated by these agents will be invaluable.

5. CONCLUSIONS AND FUTURE STUDIES

The activation and/or overexpression of c-Src has been a consistent finding in a variety of cancers; most notably, cancer of the colon, breast, liver, and pancreas (Biscardi, *et al*., 1999). Previous work from the Bonham laboratory has demonstrated that increased c-Src activity can, in part, be explained by the transcriptional overexpression of SRC in several colon cancer and breast cell lines (Dehm, *et al*., 2001; Dehm and Bonham, 2004). In an effort to determine the mechanisms responsible for SRC transcriptional overexpression in cancer cells, the regulation of the SRC promoter regions is of considerable significance. Similarly, of particular interest, SRC mRNA expression is powerfully repressed by the potent chemotherapeutic drugs, histone deacetylase inhibitors (HDIs), by a currently unknown mechanism (Kostynuik, *et al*., 2002; Dehm, *et al*., 2004).

Two disparate promoters, SRC1α and SRC1A, regulate SRC expression. The SRC1α promoter is regulated by HNF-1α and as a result is expressed primarily in tissues such as the stomach, liver, and kidney (Bonham, *et al*., 2000). The housekeeping-like SRC1A promoter is approximately 1 kb downstream of the SRC1α promoter. The SRC1A promoter is ubiquitously expressed, GC-rich and regulated by the Sp family of transcription factors and hnRNP K.

5.1. The Role of Sp3 on SRC Activity

Until recently, the Sp family factor, Sp3, was thought to be unable to activate the SRC1A promoter, however, a similar factor, Sp1, was a powerful activator of the SRC1A promoter. Recent information concerning the role of Sp3 resulted in the re-characterization of the effect of this factor on SRC expression. Through the course of these studies it was determined that the longest physiological forms of Sp3 were indeed activators of SRC1A, however, not to the same extent as Sp1. Furthermore, previous studies were utilizing Sp3 constructs that were expressing the non-physiological, N-terminal truncated form of Sp3. As such, the actual role of Sp3 within these systems

studied is circumspect due to the differing activating potential between the physiological and truncated forms of Sp3. As a result of these findings, and those of others, the true roles of the longest forms of Sp3 in transcriptional regulation are only now being realized. Moreover, comparative analyses of the putative and authentic long forms of Sp3 have identified a potential regulatory region in the N-terminal region of Sp3 that is missing in the non-physiological form of Sp3. The characterization of this region will be necessary in gleaning the complete role of the longest Sp3 isoforms in transcriptional regulation.

Aside from the importance of the longest Sp3 isoform in transcriptional regulation, the shortest isoforms have a newly identified role as potential activators of transcription. This stems from the identification of SUMOylation as an inhibitor of the short Sp3 isoforms' activation potential. Curtailing SUMOylation, either through mutation or the overexpression of a SUMO-1 protease, converted these ineffective transcription factors into potent activators. Interestingly, the same experiments to eliminate SUMOylation had little effect on the transcriptional activation by the longer isoforms of Sp3. Taken together, these results suggest that the SUMOylation of the shortest Sp3 isoforms may impede transcriptional activation, by these isoforms, through a mechanism that blocks the function of the sole activation domain present within the shortest Sp3 isoforms. A similar effect was not observed upon SUMOylation of the long isoforms potentially due to the presence of a second activation domain a significant distance away from the SUMO-1 binding site. To completely characterize the role of Sp3 on SRC activity, the proportion of which Sp3 isoforms are bound *in vivo* to the SRC1A promoter will need to be determined as well as the modification status of the isoform bound. Unfortunately, at this time, the antibodies necessary to undertake such a study are not available and only a general population of Sp3 can be identified as binding to the SRC1A promoter *in vivo*. Considering that a recent analysis of Sp factor binding sites on chromosome 21 and 22 listed a minimum of 12,000 binding sites specific for this family of factors on these chromosomes alone, suggests that theses factors are highly involved in gene regulation and essential in the characterization of promoter activity (Cawley, *et al*., 2004). Furthermore, it is through the characterization of

promoter regulation by factors such as these that the mechanism of enhanced SRC promoter activity in cancer cells may be ascertained.

5.2. HDIs repress SRC Expression

Despite the obvious dissimilarities between both SRC promoters, they have several qualities in common. Transcription is initiated from both promoters via an Inr element, and they are both repressed by the promising class of chemotherapeutic agent, histone deacetylase inhibitors (HDIs). HDIs are a particularly exciting class of chemotherapeutics due to their abilities to induce apoptosis, cell cycle arrest and terminal differentiation in neoplasms (Dokmanovic and Marks, 2005). These effects are -mediated, in part, through transcriptional upregulation and downregulation of gene expression. Historically, this differential gene expression was attributed to altered histone modifications resulting in chromatin remodelling. As HDIs inhibit the activity of histone deacetylases, a greater number of histone residues are acetylated upon treatment. Traditionally, histone acetylation is associated with transcriptionally active genomic regions due to a more relaxed chromatin conformation at these regions; therefore treatment with HDIs would increase histone acetylation leading to an "open" chromatin conformation and increased transcriptional activity. While this model is suggestive of how genes are transcriptionally activated by HDIs, it fails to address genes transcriptionally repressed by HDIs, such as SRC.

5.3. HDI Treatment Results in Similar Changes in Acetylation Status at SRC and $p21^{WAF1}$ Promoters

As a result of this model, the acetylation status of the promoter regions of two genes differentially affected by HDIs was undertaken. The $p21^{WAF1}$ promoter was used in this study as a classical example of a gene transcriptionally activated by HDIs whereas both SRC promoters were used as examples of a gene transcriptionally repressed by HDIs. Strikingly, the acetylation of histone residues at both SRC promoters and the $p21^{WAF1}$ promoter increased in response to treatment with the HDI, Trichostain A (TSA). Upon closer analysis of changes in acetylation at particular histone residues, it was identified that despite a shared repression upon TSA treatment, the SRC1α promoter exhibited changes in acetylation at discreet residues more similar to the transcriptionally activated $p21^{WAF1}$ promoter as compared to the transcriptionally

repressed SRC1A promoter. These results suggest that histone acetylation status is not an accurate predictor of transcriptional activity. Moreover, as a result of the increased histone acetylation at the SRC promoters in response to TSA, it is unlikely that chromatin remodelling is responsible for the repressive effects of HDIs at the SRC gene. Despite this compelling evidence suggesting that HDIs do not repress SRC through chromatin remodelling, to completely exclude the possibility, *in vitro* nucleosomal remodelling studies may be necessary.

5.4. SRC Transcriptional Elongation Abrogated Following TSA Treatment

As a result of findings such as those outlined above, the mechanisms behind HDI-mediated repression and activation are currently under intense study. While many suggest roles for transcription factors and HDI-responsive elements in HDI-mediated transcriptional activation, the mechanisms behind HDI-mediated repression remain elusive. HDI -mediated repression of SRC is not a result of HDI-responsive elements or protein neosynthesis, however, previous studies were able to identify potential roles for both core and proximal promoter elements in HDI-mediated SRC repression (Kostynuik, *et al*., 2002; Dehm, *et al*., 2004).

Upon closer examination, it was shown that transcription factor binding was maintained *in vivo* upon treatment with TSA. RNA Polymerase II (RNA Pol. II) occupancy was also maintained at both SRC promoter regions despite TSA treatment. Upon tracking RNA Pol. II occupancy along the SRC gene, however, RNA Pol. II occupancy at the 3' UTR decreased in response to TSA treatment. Interestingly, the amount of time that elapsed before a decrease in Pol. II occupancy could be observed at the 3'UTR correlated well with the amount of time required to observe TSA -mediated effects on RNA Pol. II occupancy at the promoter region at the most distal ends of the gene. Essentially, it appeared as though RNA Pol. II was sequestered to the SRC promoter regions. To identify if TSA was abrogating transcriptional initiation or elongation, the phosphorylation status of RNA Pol. II upon TSA treatment was identified. The phosphorylation of RNA Pol. II suggested that while transcription initiation was occurring, a mature transcript was not formed due to the absence of RNA Pol II at the 3' UTR. As such, a role for abrogated transcriptional elongation in HDI -mediated SRC repression was identified. In support of these observations, transient

transfection analysis of SRC reporter constructs were also susceptible to HDI -mediated repression. Again, taken together, these results all suggest that a region encompassed in the reporter construct, downstream of the transcriptional start site is responsive to HDI -mediated repression of SRC.

5.5. Class II HDACs May be Required to Maintain SRC mRNA Levels

In contrast to HDI -mediated repression of SRC via abrogated transcriptional elongation, other groups have identified that an HDAC is required for transcriptional activation at several promoters that are interferon, virus or hypoxia induced. Therefore, in these systems, HDIs mediate transcriptional repression through the inhibition of the HDAC essential to transcriptional activation (Rascle, *et al*., 2003; Xu, *et al*., 2003; Chang, *et al*., 2004; Kato, *et al.*, 2004; Klampfer, *et al*., 2004; Sakamoto, *et al.*, 2004; Nusinzon and Horvath, 2006). Despite innate differences between SRC and these systems requiring an HDAC, it was discovered that SRC expression is also dependent on a class II HDAC. In contrast to that observed with induced genes, SRC transcriptional activation was not enhanced by the presence of an HDAC. Indeed, the overexpression of an individual class I or II HDAC often resulted in decreased, not enhanced, SRC promoter activity. However, siRNA -mediated knockdowns of HDAC5, 6, 7 or 10 resulted in an eventual decrease in SRC transcript levels. Recent studies have identified that HDIs are capable of disrupting phosphatase/HDAC complexes, resulting in aberrant phosphatase activity throughout the cell (Chen, *et al*., 2005; Alao, *et al*., 2006). One example is the disruption of the HDAC6/PP1 complex. Interestingly, both the positive elongation factor P-TEFb and the CTD of RNA Pol. II are among the many targets of PP1 (Washington, *et al*., 2002; Ammosova, *et al*., 2005). The phosphorylation of either of these targets is critical in maintaining productive transcriptional elongation to yield a mature mRNA transcript. Therefore if HDI treatment inhibited HDAC6 thus resulting in aberrant PP1 phosphatase activity targeting either or both of these critical components of elongation, expression would be repressed, as a mature transcript could not be produced. Interestingly, these events could be easily be occurring downstream of the SRC promoters to impede transcriptional elongation.

5.6. Future Studies

Ideally, to validate this model several studies are required. To identify what role if any the above factors have in HDI -mediated repression there are a host of inhibitors available including: an inhibitor specific for HDAC6, tubacin, an inhibitor specific for P-TEFb, DRB, and a whole arsenal of PP1 inhibitors. In the case of P-TEFb, it would be expected that if P-TEFb is inhibited thus preventing SRC transcriptional elongation, treatment with DRB would result in SRC repression in a manner similar to what is observed with HDI--mediated repression. If tubacin is used to inhibit HDAC6 and SRC is still repressed than we have identified that the repression of HDAC6 activity is responsible for HDI -mediated SRC repression. Moreover, with the exception of TSA and SAHA, many HDIs are specific for one class of HDAC versus another therefore the potential involvement of a particular HDAC could be elucidated through the systematic investigation of which HDI is capable of repressing SRC. Finally, if PP1 is mediating the elongation abrogating effects on SRC expression, the treatment of cells with a PP1 inhibitor after treatment of cells with TSA should rescue SRC expression. Regardless, of the actual outcome of the inhibitor studies, the information gleaned would be invaluable in further characterizing HDI -mediated SRC repression.

Unfortunately, there are not ample methods for studying transcriptional elongation *in vivo*. ChIP assays are one method that is frequently utilized, however, with this technique it is difficult to identify the precise sequence where elongation is attenuated. Despite these difficulties, a great deal could be determined through this technique. Many groups have had success with antibodies specific for P-TEFb, NELF and DSIF. Moreover, if an amplicon downstream of the SRC promoters was analysed, it may be possible to observe greater enrichment in serine 2 CTD RNA Pol. II phosphorylation and any changes in this phosphorylation -mediated by HDI treatment could be quantified. Alternate techniques, such as RNAse protection assays (RPAs) and RT-PCR at particular regions of the transcript, are also possible; however, these techniques could prove to be overly problematic depending on where along the transcript elongation is terminated. For example, if elongation is curtailed within 100 bases of the promoter region, the transcript may be too short for identification as well as

may degrade too quickly due to a lack of stabilizing co-transcriptional processing such as 5' methyl-guanine capping.

Despite the technical difficulties that must be addressed, the role of transcriptional elongation and class II HDAC(s) in HDI -mediated repression of SRC should be clarified. In doing so, the mechanism of repression of the SRC proto-oncogene by the powerful chemotherapeutics, HDIs, may be determined. Given that approximately half of all genes affected by HDIs are repressed, a mechanism for this repression is of extreme importance in the development of future chemotherapeutics.

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